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## NOVEL PROTEINS AND METHODS FOR PRODUCING THE PROTEINS

### Field of the invention

This invention relates to a novel protein, osteoclastogenesis inhibitory factor (OCIF), and methods for producing the protein.

### Background of the invention

Human bones are always remodelling by the repeated process of resorption and reconstitution. [In the process, osteoblasts] ^Osteoblasts and osteoclasts are considered to be the cells mainly responsible for bone formation and bone resorption, respectively. A typical example of ^a disease caused by [the progression of] abnormal bone metabolism is osteoporosis. [The disease] ^Osteoporosis is known to [be provoked] ^result [by the condition in which] ^when bone resorption by osteoclasts exceeds bone formation by osteoblasts, but the mechanism of osteoporosis has not yet been completely elucidated. Osteoporosis causes ^bone pain [in the bone] and makes [the bone] ^bones [fragile, leading to fracture. Since osteoporosis increases the number of] ^fragile, leading to fracture, particularly in elderly patients. [bedridden old people, it has] ^Osteoporosis has therefore become a social issue with the increasing number of [old] ^elderly people ^in the population. Therefore, [efficacious] ^effective drugs for the treatment of the disease are expected to be developed. Bone mass reduction caused by [the] abnormal bone metabolism is thought to be prevented by inhibiting bone resorption, improving bone formation, or improving the [balanced] ^balance of bone metabolism.

Bone formation is [expected to be] promoted by stimulating growth, differentiation, or activation of osteoblasts. Many cytokines [are reported] ^reportedly [to] stimulate growth or [differentation] ^differentiation of osteoblasts , i.e. fibroblast growth

factor (FGF) (Rodan S.B. et al., Endocrinology vol.121, p1917, 1987), insulin-like growth factor-I (IGF-I) (Hock J.M. et al., Endocrinology vol. 122, p254, 1988), insulin-like growth factor-II (IGF-II) (McCarthy T. et al., Endocrinology vol.124, p301, 1989), Activin A (Centrella M. et al., [Mol, Cell,] Mol. Cell, Biol. vol. 11, p250, 1991), Vasculotropin (Varonique M. et al., Biochem. Biophys. Res. Commun. vol. 199, p380, 1994), and bone morphogenetic protein (BMP) (Yamaguchi, A. et al., J. Cell Biol. vol. 113, p682, 1991, Sampath T.K. et al., J. Biol. Chem. vol.267, p20532, 1992, and Knutsen R. et al., Biochem. Biophys. Res. Commun. vol.194, p1352, [1993.] 1993).

On the other hand, cytokines which [inhibits] inhibit differentiation and/or maturation of osteoclasts [have been paid attention] and have also been intensively studied. Transforming growth factor-$\beta$ (Chenu C. et al., Proc. Natl. Acad. Sci. USA, vol.85, p5683, 1988) and interleukin-4 (Kasano K. et al., Bone-Miner., vol. 21, p179, 1993) [are found to] inhibit the differentiation of osteoclasts. Calcitonin (Bone-Miner., vol.17, p347, 1992), [Macrophage] macrophage colony-stimulating factor (Hattersley G. et al. J. Cell. Physiol. vol.137, p199, 1988), interleukin-4 (Watanabe, K. et al., Biochem. Biophys. Res. Commun. vol. 172, p1035, 1990), and interferon-$\gamma$ (Gowen M. et al., J. Bone Miner. Res., vol.1, p469, 1986) [are found to] inhibit bone resorption by osteoclasts.

These cytokines are expected to be [efficacious] effective drugs for improving bone mass reduction by stimulating bone formation and/or by inhibiting bone resorption. [The cytokines] Cytokines such as [insulin like] insulin-like growth factor-I and bone morphogenetic proteins [are now] have been investigated in clinical trials for their

effectiveness for treating
[effects in treatment of] patients with bone diseases. Calcitonin is already used [as a drug to care] osteoporosis and to diminish pain in osteoporosis. patients

Examples of drugs now clinically utilized for the treatment of bone diseases and for shortening the treatment period are dihydroxyvitamine $D_3$, vitamin $K_2$, calcitonin and its derivatives, hormones such as estradiol, ipriflavon, and calcium [preparations.] However, these drugs do not provide satisfactory therapeutic effects, and novel drug substances [have been] expected to be developed. [As mentioned,] bone metabolism is [controlled] in the balance between bone resorption and bone [formation. Therefore,] cytokines which inhibit osteoclast differentiation and/or maturation are expected to be developed as drugs for the treatment of bone diseases such as osteoporosis.

Summary
[Disclosure] of Invention

[This invention was initiated from the view point described above. The] The purpose of this invention is to offer both a novel factor, termed osteoclastogenesis inhibitory factor (OCIF), and a procedure to produce the factor efficiently.

The inventors have intensively searched for osteoclastogenesis inhibitory factors in human embryonic fibloblast IMR-90 (ATCC CCL186) conditioned medium and have found a novel osteoclastogenesis inhibitory factor (OCIF) which inhibits differentiation and/or maturation of osteoclasts.

The inventors have established a method for accumulating the protein to a high concentration by culturing IMR-90 cells [using] on alumina ceramic pieces, which function as [the] cell adherence matrices.

The inventors have also established an efficient method for isolating the protein, OCIF, from the IMR-90 conditioned medium using the following sequential column chromatography, ion-exchange, heparin affinity, cibacron-blue affinity, and reverse phase.

The inventors, based on the amino acid sequence of the purified natural OCIF, successfully cloned a cDNA encoding this protein. The inventors established also a procedure to produce this protein which inhibits differentiation of osteoclasts. This invention concerns a protein which is produced by human lung fibroblast cells, has molecular weights in SDS-PAGE of 60 KD in the reducing conditions and 120 KD under the non-reducing conditions, has affinity for both cation-exchange resins and heparin, reduces its activity to inhibit differentiation and maturation of osteoclasts if treated for 10 minutes at 70 ℃ or for 30 minutes at 56 ℃, and lose its activity to inhibit differentiation and maturation of osteoclasts by the treatment for 10 minutes at 90 ℃. The amino acid sequence of the protein OCIF which is described in the present invention is clearly different from any of know factors inhibiting formation of osteoclasts.

The invention includes a method to purify OCIF protein, comprising ; (1) culturing human fibroblasts, (2) applying the conditioned medium to a heparin column to obtain the adsorbed fraction, (3) purifying the OCIF protein using a cation-exchange column, (4) purifying the OCIF protein using a heparin affinity column, (5) purifying the OCIF protein using a cibacron blue affinity column, (6) isolating the OCIF protein using reverse-phase column chromatography. Cibacron blue F3GA coupled to a carrier made of synthetic

hydrophilic polymers, for example.
[hydrophilic polymers is an example of materials used to prepare Cibacron blue columns.] These columns are conventionally called "blue colomns". columns

The invention includes a method for [accumulating producing the] OCIF protein [to a] in high concentration by culturing human fibroblasts using alumina ceramic pieces as the cell-adherence matrices.

Moreover, the inventors determined the amino acid sequences of [the] peptides derived from OCIF, designed the oligonucleotide primers based on these amino acid sequences, and obtained cDNA fragments encoding OCIF from a cDNA library of IMR-90 cells. The full length OCIF cDNA encoding the OCIF protein is cloned from a cDNA library using an OCIF DNA fragment as a probe. The OCIF cDNA containing the entire coding region is inserted into an expression vector. Recombinant OCIF can be produced by expressing the OCIF cDNA, containing the entire coding region, in mammalian cells or bacteria. *See Sheet 5A attached

Detailed description of the invention

The OCIF protein of the present invention can be isolated from human fibroblast conditioned medium with high yield. The procedure to isolate OCIF is based on ordinary techniques for purifying proteins from biomaterials, in accordance with the physical and chemical properties of OCIF protein. For example, concentrating [procedure includes] procedures include ordinary biochemical techniques such as ultrafiltration, [lyophylization] lyophilization, and dialysis. Purifying [procedure includes] procedures include combinations of several chromatographic techniques for purifying proteins such as ion-exchange column chromatography, affinity column chromatography, gel filtration column chromatography, hydrophobic column chromatography, reverse phase column chromatography, and preparative gel electrophoresis. The human [fibroblast used for production of the OCIF protein is preferably IMR-90. A] fibroblasts used for the production of OCIF protein are preferably IMR-90 cells. A method for producing [the] IMR-90 conditioned medium is preferably a process comprising, adhering human embryonic fibroblast IMR-90 cells to alumina

Text to be inserted before "Detailed description of the invention:

Brief Description of the Figures

Figure 1 shows the elution pattern of crude OCIF protein (HiLoad-Q/FF pass-through fraction; sample 3) from a HiLoad-S/HP column.

Figure 2 shows the elution pattern of crude OCIF protein (Heparin-5PW fraction; sample 5) from a blue-5PW column.

Figure 3 shows the elution pattern of OCIF protein (blue-5PW fraction 49 to 50) from a reverse-phase column.

Figures 4A and 4B show the SDS-PAGE of isolated OCIF proteins under reducing or non-reducing conditions. Description of the lanes:

lane 1, 4: molecular weight marker proteins;
lane 2, 5: OCIF protein of peak 6 in Figure 3;
lane 3, 6: OCIF protein of peak 7 in Figure 3.

Figure 5 shows the elution pattern of peptides obtained by the digestion of pyridylethylated OCIF protein digested with lysylendopeptidase, on a reverse-phase column.

Figure 6 shows the SDS-PAGE of isolated natural (n) OCIF protein and recombinant (r) OCIF proteins under non-reducing conditions. rOCIF(E) and rOCIF(C) proteins were produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 1: molecular weight marker proteins;
lane 2: a monomer type nOCIF protein;
lane 3: a dimer type nOCIF protein;
lane 4: a monomer type rOCIF(E) protein;
lane 5: a dimer type rOCIF(E) protein;
lane 6: a monomer type rOCIF(C) protein;
lane 7: a dimer type rOCIF(C) protein.

Figure 7 shows the SDS-PAGE of isolated natural (n) OCIF proteins and recombinant (r) OCIF proteins under reducing conditions. rOCIF(E) and rOCIF(C) were produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 8: molecular weight marker proteins;
lane 9: a monomer type nOCIF protein;
lane 10: a dimer type nOCIF protein;
lane 11: a monomer type rOCIF(E) protein;
lane 12: a dimer type rOCIF(E) protein;
lane 13: a monomer type rOCIF(C) protein;
lane 14: a dimer type rOCIF(C) protein.

Figure 8 shows the SDS-PAGE of isolated natural (n) OCIF proteins and recombinant (r) OCIF proteins from which N-linked sugar chains were removed under reducing conditions. rOCIF(E) and rOCIF(C) are rOCIF proteins produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 15: molecular weight marker proteins;
lane 16: a monomer type nOCIF protein;
lane 17: a dimer type nOCIF protein;
lane 18: a monomer type rOCIF(E) protein;
lane 19: a dimer type rOCIF(E) protein;
lane 20: a monomer type rOCIF(C) protein;
lane 21: a dimer type rOCIF(C) protein.

Figure 9 shows a comparison of OCIF and OCIF2 amino acid sequences.

Figure 10 shows a comparison of OCIF and OCIF3 amino acid sequences.

Figure 11 shows a comparison of OCIF and OCIF4 amino acid sequences.

Figure 12 shows a comparison of OCIF and OCIF5 amino acid sequences.

Figure 13 shows a standard curve determining OCIF protein concentration by an EIA employing anti-OCIF polyclonal antibodies.

Figure 14 shows a standard curve determining OCIF protein concentration by an EIA employing anti-OCIF monoclonal antibodies.

Figure 15 shows the effect of r-OCIF protein on model rats with osteoporosis.

ceramic pieces in roller-bottles, using DMEM medium supplemented with 5 % new born calf serum for the cell culture, and cultivating the cells in roller-bottles for 7 to 10 days by stand cultivation. CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) is prefarably added to the buffer as a detergent in the purification steps of OCIF protein.

The OCIF protein of the instant invention can be initially obtained as a heparin binding basic OCIF fraction by applying the culture medium to a heparin column (Heparin-Sepharose CL-6B, Pharmacia), eluting with 10 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl, and then by applying the OCIF fraction to a Q • anion-exchange column (HiLoad-Q/FF, Pharmacia), and collecting non-adsorbed fraction. OCIF protein can be purified by subjecting the obtained OCIF fraction to purification on a S • cation-exchange column (HiLoad-S/FF, Pharmacia), a heparin column (Heparin-5PW, TOSOH), Cibacrone Blue column (Blue-5PW, TOSOH), and a reverse-phase column (BU-300 C4, Perkin Elmer) and the material is defined by the previously described properties.

The present invention relates to a method of cloning cDNA encoding the OCIF protein based on the amino acid sequence of natural OCIF and a method of obtaining recombinant OCIF protein that inhibits differentiation and/or maturation of osteoclasts. The OCIF protein is purified according to the method described in the present invention and is treated with endopeptidase (for example, lysylendopeptidase). The amino acid sequences of the peptides produced by the digestion are determined and the mixture of oligonucleotides

that can encode each internal amino acid sequence [was systhesized.] is synthesized. The OCIF cDNA fragment is obtained by PCR (preferably RT-PCR, reverse transcriptase PCR) using the oligonucleotide mixtures described above as primers. The full length OCIF cDNA encoding the OCIF protein is cloned from a cDNA library using [the] [obtained] an OCIF DNA fragment as a probe. The OCIF cDNA containing the entire coding region is inserted into an expression vector. [The recombinant] Recombinant OCIF can be produced by expressing the OCIF cDNA, containing the entire coding region, in mammalian cells or bacteria.

The present invention relates to the novel proteins OCIF2, OCIF3, OCIF4, and OCIF5 that are variants of OCIF and have the activity described above. These OCIF variants are obtained from the cDNA library constructed with IMR-90 poly(A) + RNA [by hybridization] using the OCIF cDNA fragment as a hybridization probe. Each of the OCIF variant cDNAs containing the entire coding region is inserted into an expression vector. Each recombinant OCIF variant protein can be produced by expressing each of the OCIF variant cDNAs, containing the entire coding region, in [the] conventional hosts. Each recombinant OCIF variant protein can be purified according to the method described in this invention. Each recombinant OCIF variant protein has [an] the ability to inhibit osteoclastogenesis.

The present invention further includes OCIF mutants. They are substitution mutants comprising the replacement of one cysteine residue, possibly involved in dimer [formation with serine residue, and] formation, with a serine residue or various deletion mutants of OCIF. Substitutions or deletions are introduced into the OCIF cDNA using

polymerase chain reaction (PCR) or by restriction enzyme digestion. Each of these mutated OCIF cDNAs is inserted into a vector containing an appropriate promoter for gene expression. The resultant expression vector for each of the OCIF mutants is transfected into eukaryotic cells such as mammalian cells. Each of OCIF mutants can be obtained and purified from the conditioned media of the transfected cells.

The present invention provides polyclonal antibodies and a method to quantitatively determine OCIF concentration using these polyclonal antibodies.

As antigens (immunogens), natural OCIF obtained from IMR-90 conditioned medium, recombinant OCIF produced by such hosts as microorganisms and eukaryotes using OCIF cDNA, synthetic peptides designed based on the amino acid sequence of OCIF, or peptides obtained from OCIF by partial digestion can be used. Anti-OCIF polyclonal antibodies are obtained by immunizing appropriate mammals with the antigens, in combination with adjuvants for immunization if necessary, purifying from the serum by the ordinary purification methods. The anti-OCIF polyclonal antibodies which are labelled with rasioisotopes or enzymes can be used in radio-immunoassay (RIA) system or immunoassay (EIA) system. By using these assay systems, the concentrations of OCIF in biological materials such as blood and ascites and cells-culture medium can be easily determined.

The antibodies in the present invention can be used in radio immunoassay (RIA) or enzyme immunoassay (EIA). By using these assay systems, the concentration of OCIF in biological materials such as blood and ascites can

be easily determined.

The present invention provides novel monoclonal antibodies and a method to quantitatively determine OCIF concentration using these monoclonal antibodies.

Anti-OCIF monoclonal antibodies can be produced by the conventional method using OCIF as an antigen. Native OCIF obtained from the culture medium of IMR-90 cells and recombinant OCIF produced by such hosts as microorganisms and eukaryotes using OCIF cDNA can be used as antigens. Alternatively, synthesized peptides designed based on the amino acid sequence of OCIF and peptides obtained from OCIF by partial digestion can be also used as antigens. Immunized lymphocytes obtained by immunization of mammals with the antigen or by an in vitro immunization method were fused with myeloma of mammals to obtain hybridoma. The hybridoma clones secreting antibody which recognizes OCIF were selected from the hybridomas obtained by the cell fusion. The desired antibodies can be obtained by cell culture of the selected hybridoma clones. In preparation of hybridoma, small animals such as mice or rats are generally used for immunization. To immunize, OCIF is suitably diluted with a saline solution (0.15 M NaCl), and is intravenously or intraperitoneally administered with an adjuvant to animals for 2 -5 times every 2 -20 days. The immunized animal was killed three days after final immunization, the spleen was taken out and the splenocytes were used as immunized B lymphocytes.

Mouse myeloma cell lines for cell fusion with the immunized B lymphocytes include, for example, p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, FO, p3x63

Ag8.653, and S194 cells. The rat cell line R-210 may also be used. Alternatively, human B lymphocytes

[Ag8.653, and S194. Rat R-210 cell line may also be used. Human B lymphocytes] [are] immunized by an in vitro immunization method [and] are fused with human myeloma [cell line] cells or EB virus transformed human B lymphocytes [which are used] [as a parent cell line for cell fusion, to produce human type antibody.] to produce human type antibodies.

Cell fusion of [the] immunized B lymphocytes and myeloma [cell line] cells is carried out principally by [the] conventional methods. For example, the method of Koehler G. et al. (Nature 256, 495-497, 1975) is generally [used, and also] used. Alternatively, an electric pulse method can be [applied to cell fusion.] used. The immunized B lymphocytes and transformed B cells are mixed at conventional ratios and a cell culture medium without FBS containing polyethylene glycol is generally [used for cell fusion. The B lymphocytes fused with myeloma cell lines are] used to fuse the cells. The fusions products are cultured in HAT selection medium containing FBS to select [hybridoma.] hybridomas.

[For screening of hybridoma producing anti-OCIF antibody, EIA, plaque] An EIA, plaque assay, Ouchterlony, or agglutination assay can be [principally used.] used to screen for hybridomas producing anti-OCIF antibodies. [Among them, EIA is simple and easy to operate] EIA is a simple assay which is easy to perform with sufficient accuracy and is [generally used.] therefore generally used. By EIA using purified OCIF, the desired antibody can be selected easily and accurately. Thus obtained hybridoma [can be cultured by the] using EIA and purified OCIF. Hybridomas obtained there conventional [method] methods of cell culture and frozen for stock if necessary. The antibody can be produced by culturing hybridoma [using the] cells using ordinary cell culture [method] methods or by transplanting hybridoma cells intraperitoneally [to] into live animals. The antibody can be purified by [the] ordinary purification methods such as salt precipitation, gel filtration, and affinity chromatography. The [obtained] antibody obtained specifically reacts with OCIF and can be used [for determination of] to determine OCIF concentration and [for purification of OCIF.] to purify OCIF protein. The antibodies of the

present invention recognize epitopes of OCIF and have high affinity [to] ^ for OCIF. Therefore, they can be used for the construction of EIA. [By (using) this assay system,] the concentration of OCIF in biological materials such as [blood and ascites can be easily determined.] ^ blood and ascites.

[The agents used for treating bone diseases that contain OCIF as an effective ingredient are provided by the present invention.] ^ The present invention provides agents, containing OCIF as an ingredient, that are useful for treating bone diseases. Rats were subjected to denervation of ^ the left forelimb. Test compounds were administered daily after surgery for 14 days. After 2 weeks ^ of treatment, the animals were sacrificed and their forelimbs were dissected. Thereafter bones were tested for mechanical strength by ^ the three point bending method. OCIF improved ^ the mechanical strength of bone in a dose dependent manner.

The OCIF protein of the invention is useful as a pharmaceutical [ingredients] ^ ingredient for treating or improving decreased bone mass in ^ such as bone diseases osteoporosis, [bone diseases such as] rheumatism, osteoarthritis, and abnormal bone metabolism in multiple myeloma. [The] OCIF protein is also useful as an antigen [to establish] ^ in the immunological diagnosis of [the] ^ bone diseases. Pharmaceutical preparations containing [the] OCIF protein as an active [ingredients] ^ ingredient are formulated and can be orally or parenterally administered. The preparation contains the OCIF protein of the present invention as an [efficacious] ^ effective ingredient and is safely administered to [human] ^ humans and animals. Examples of [the] pharmaceutical preparations include compositions for injection or intravenous drip, suppositories, nasal preparations, sublingual preparations, and tapes for percutaneous absorption. The pharmaceutical preparation for injection can

be prepared by mixing [the] pharmacologically [efficacious] amount of OCIF protein and pharmaceutically acceptable [carriers.] The carriers are vehicles and/or activators, e.g. amino acids, saccharides, cellulose derivatives, and other organic and inorganic compounds, which are generally added to active ingredients. When the OCIF protein is mixed with the vehicles and/or [activators to prepare injections, pH adjuster, buffer, stabilizer,] solubilizing agent, etc. can be added, if necessary.

[Brief description of the figures

Figure 1 shows the elution pattern of crude OCIF protein (Hiload-Q/FF pass-through fraction ; sample 3) from a Hiload-S/HP column.

Figure 2 shows the elution pattern of crude OCIF protein (heparin-5PW fraction ; sample 5) from a blue-5PW column.

Figure 3 shows the elution pattern of OCIF protein (blue-5PW fraction 49 to 50) from a reverse-phase column.

Figure 4 shows the SDS-PAGE of isolated OCIF proteins under reducing conditions or non-reducing conditions.

Description of the lanes,

lane 1,4 ; molecular weight marker proteins

lane 2,5 ; OCIF protein of peak 6 in figure 3

lane 3,6 ; OCIF protein of peak 7 in figure 3

Figure 5 shows the elution pattern of peptides obtained by the digestion of pyridyl ethylated OCIF protein digested with lysylendopeptidase, on a reverse-phase column.]

Figure 6 shows the SDS-PAGE of isolated natural(n) OCIF protein and recombinant(r) OCIF proteins under non-reducing conditions. rOCIF(E) and rOCIF(C) were produced in 293/EBNA cells and in CHO cells, respectively.

Description of the lanes,

lane 1 ; molecular weight marker proteins

lane 2 ; a monomer type nOCIF protein

lane 3 ; a dimer type nOCIF protein

lane 4 ; a monomer type rOCIF(E) protein

lane 5 ; a dimer type rOCIF(E) protein

lane 6 ; a monomer type rOCIF(C) protein

lane 7 ; a dimer type rOCIF(C) protein

Figure 7 shows the SDS-PAGE of isolated natural(n) OCIF proteins and recombinant (r) OCIF proteins under reducing conditions. rOCIF(E) and rOCIF(C) were produced in 293/EBNA cells and in CHO cells, respectively.

Description of the lanes,

lane 8 ; molecular weight marker proteins

lane 9 ; a monomer type nOCIF protein

lane 10 ; a dimer type nOCIF protein

lane 11 ; a monomer type rOCIF(E) protein

lane 12 ; a dimer type rOCIF(E) protein

lane 13 ; a monomer type rOCIF(C) protein

lane 14 ; a dimer type rOCIF(C) protein

Figure 8 shows the SDS-PAGE of isolated natural(n) OCIF proteins and recombinant(r) OCIF proteins from which N-linked sugar chains were removed

under reducing conditions. rOCIF(E) and rOCIF(C) are rOCIF protein produced in 293/EBNA cells and in CHO cells, respectively.

Description of the lanes,

lane 15 ; molecular weight marker proteins

lane 16 ; a monomer type nOCIF protein

lane 17 ; a dimer type nOCIF protein

lane 18 ; a monomer type rOCIF(E) protein

lane 19 ; a dimer type rOCIF(E) protein

lane 20 ; a monomer type rOCIF(C) protein

lane 21 ; a dimer type rOCIF(C) protein

Figure 9 shows comparison of amino acid sequences between OCIF and OCIF2.

Figure 10 shows comparison of amino acid sequences between OCIF and OCIF3.

Figure 11 shows comparison of amino acid sequences between OCIF and OCIF4.

Figure 12 shows comparison of amino acid sequences between OCIF and OCIF5.

Figure 13 shows standard curve for determination of OCIF protein concentration by an EIA employing anti-OCIF polyclonal antibodies.

Figure 14 shows standard curve for determination of OCIF protein concentration by an EIA employing anti-OCIF monoclonal antibodies.

Figure 15 shows the effect of rOCIF protein on osteoporosis.

Best Mode for Carrying Out the Invention

The present invention will be further explained by the following examples, however, though the scope of the invention is not restricted to the thereto. examples.

EXAMPLE 1

Preparation of a conditioned medium of human fibroblast IMR-90

Human fetal lung fibroblast IMR-90 (ATCC-CCL186) cells were cultured on alumina ceramic pieces (80 g) (alumina: 99.5%, manufactured by Toshiba Ceramic K.K.) in DMEM medium (manufactured by Gibco BRL Co.) supplemented with 5% CS and 10mM HEPES buffer (500 ml/roller bottle) at 37℃ [under] ^in the presence of 5% $CO_2$ for 7 to 10 days using 60 roller bottles (490 $cm^2$, 110 x 171mm, manufactured by [Coning] ^Corning Co.) in static culture. The conditioned medium was harvested, and a fresh medium was added to the roller bottles. About 30L of IMR-90 conditioned medium per batch culture was obtained. The conditioned medium was designated as sample 1.

EXAMPLE 2

Assay method for osteoclast development inhibitory activity

Osteoclast development inhibitory activity was assayed by measuring tartrate-resistant acid phosphatase(TRAP) activity according to the methods of M. Kumegawa et.al (Protein ・Nucleic Acid・Enzyme, vol.34 p999, 1989) and N. Takahashi [et.al] ^et al. (Endocrynology, vol.122, p1373, 1988 ) with modifications. Briefly, bone marrow cells obtained from ^a 17 day-old mouse were suspended in α-MEM (manufactured by GIBCO BRL Co.) containing 10% FBS, [$2x10^{-8}M$] ^$1x10^{-8}M$ of activated vitamin [$D_3$, and each test sample,] ^$D_3$ and a test sample and were inoculated [to] ^into each well of ^a 96-well plate at a cell density of $3x10^5$ cells/0.2 ml/well. The plates were incubated for 7 days at 37℃ in humidified 5%$CO_2$. Cultures were [further] ^maintained [continued] by replacing 0.16 ml of old medium with the same volume of fresh

medium on day 3 and day 5 after starting cultivation. On day 7, after washing the plates with phosphate buffered saline, cells were fixed with ethanol/acetone (1:1) for 1 min. at room temperature, and then osteoclast development was tested by determining for phosphatase activity using a kit (Acid Phosphatase, Leucocyte, Catalog No. 387-A, manufactured by Sigma Co.). The decrease of TRAP positive cells was taken as an indication of OCIF activity.

EXAMPLE 3

Purification of OCIF

i) Heparin Sepharose CL-6B column chromatography

The 90L of IMR-90 conditioned medium (sample 1) was filtrated with 0.22 $\mu$ membrane filter (hydrophilic Milidisk, 2000 $cm^2$, Milipore Co.), and was divided into three portions. Each portion (30 l) was applied to a heparin Sepharose CL-6B column (5 x 4.1 cm, Pharmacia Co.) equilibrated with 10mM Tris-HCl containing 0.3M NaCl, pH 7.5. After washing the column with 10mM Tris-HCl, pH 7.5 at a flow rate of 500 ml/hr., heparin Sepharose CL-6B adsorbent protein fraction was eluted with 10mM Tris-HCl, pH 7.5, containing 2M NaCl. The fraction was designated as sample 2.

ii) HiLoad-Q/FF column chromatography

The heparin Sepharose-adsorbent fraction (sample 2) was dialyzed against 10mM Tris-HCl, pH 7.5, supplemented with CHAPS to a final concentration of 0.1%, incubated at 4 ℃ overnight, and divided into two portions. Each

portion was then applied to an anion-exchange column (HiLoad-Q/FF, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5 to obtain a non-adsorbent fraction (1000 ml). The fraction was designated as sample 3.

iii) HiLoad-S/HP column chromatography

The HiLoad-Q non-adsorbent fraction (sample 3) was applied to a cation-exchange column (HiLoad-S/HP, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 50 mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient from 0 to 1 M NaCl at a flow rate of 8 ml/min for 100 min. and fractions (12 ml) were collected. Every ten fractions from number 1 to 40 were pooled to form one portion. Each 100 µl of the four portions was tested for OCIF activity. OCIF activity was observed in fractions from 11 to 30 (as shown in Figure 1). Fractions 21 to 30, which had higher specific activity, were pooled and designated sample 4.

iv) Heparin-5PW affinity column chromatography

One hundred and twenty ml of HiLoad-S fraction from 21 to 30 (sample 4) was diluted with 240 ml of 50 mM Tris-HCl, 0.1% CHAPS, pH 7.5, and applied to a heparin-5PW affinity column (0.8 x 7.5 cm, Tosoh Co.) which was equilibrated with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient from 0 to 2M NaCl at a flow rate of 0.5ml/min for 60 min. and fractions

(0.5 ml) were collected. Fifty μl [was] removed from each fraction to test for OCIF activity. The active fractions, eluted with 0.7 to 1.3M NaCl [was] were pooled and [was designated as] designated sample 5.

v) Blue 5PW affinity column chromatography

Ten ml of sample 5 [was] were diluted with 190 ml of 50mM Tris-HCl, 0.1% CHAPS, pH 7.5 and applied to a blue-5PW affinity column, (0.5x5 cm, Tosoh Co.) which was equilibrated with 50mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 50mM Tris-HCl, 0.1% CHAPS, pH7.5, the adsorbed protein was eluted with a 30 ml linear gradient from 0 to 2M NaCl at a flow rate of 0.5 ml/min., and fractions (0.5 ml) were collected. Using 25 μl of each fraction, OCIF activity was evaluated. [The fractions number] Fractions 49 to 70, eluted with 1.0-1.6M NaCl, had OCIF activity.

vi) Reverse phase column chromatography

The blue 5PW fraction obtained by collecting fractions [from 49 to] 49 and 50 was acidified with 10μl of 25% TFA and applied to a reverse phase C4 column (BU-300, 2.1x220mm, manufactured by Perkin-Elmer) which was equilibrated with 0.1% of TFA and 25% [of] acetonitrile. The adsorbed protein was eluted with a linear gradient from 25 to 55% acetonitrile at a flow rate of 0.2 ml/min. for 60 min., and each protein peak was collected (Fig. 3). One hundred μl of each peak fraction was tested for OCIF activity, and [peak 6 and the peak] peaks 6 and 7 had OCIF activity. The result was shown in Table 1.

Table 1

OCIF activity eluted from reverse phase C4 column

| Sample | Dilution | | | |
|---|---|---|---|---|
| | 1/40 | 1/120 | 1/360 | 1/1080 |
| Peak 6 | ++ | ++ | + | - |
| Peak 7 | ++ | + | - | - |

[ ++ means OCIF activity inhibiting osteoclast development more than 80%, + means OCIF activity inhibiting osteoclast development between 30% and 80%, and - means no OCIF activity.]

EXAMPLE 4

Molecular weight of OCIF protein

The two protein peaks [(6 and 7) with OCIF activity (peaks 6 and 7)] were subjected to SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Briefly, 20 $\mu$l of each peak fraction was concentrated under vacuum and dissolved in 1.5 $\mu$l of 10mM Tris-HCl, pH 8, 1mM EDTA, 2.5% SDS, 0.01% bromophenol blue, and incubated at 37℃ overnight under non-reducing conditions or under reducing conditions (with 5% of 2-mercaptoethanol). Each 1.0 $\mu$l of sample was then analyzed by SDS-polyacrylamide gel electrophoresis with a gradient gel of 10-15% acrylamide (Pharmacia Co.) and an electrophoresis-device (Fast System, Pharmacia Co.). The following molecular weight marker proteins were used to calculate molecular weight : phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.0 kD), and lactalbumin (14.4 kD). After

electrophoresis, protein bands were visualized by silver stain using Phast Silver Stain Kit. The results were shown in Fig. 4.

A protein band with an apparent 60 KD was detected in the peak 6 protein under both reducing and non-reducing conditions. A protein band with an apparent 60 KD was detected under reducing conditions and a protein band with an apparent 120 KD was detected under non-reducing conditions in the peak 7 protein. Therefore, the protein of peak 7 was considered to be a homodimer of the protein of peak 6.

EXAMPLE 5

Thermostability of OCIF

Twenty $\mu$l of sample from the blue-5PW fractions 51 and 52 was diluted to 30$\mu$l with 10 mM phosphate buffered saline, pH 7.2, and incubated for 10 min. at 70℃ or 90 ℃, or for 30 min. at 56℃. The heat-treated samples were tested for OCIF activity. The results were shown in Table 2.

Table 2

Thermostability of OCIF

| Sample | Dilution | | |
|---|---|---|---|
| | 1/300 | 1/900 | 1/2700 |
| untreated | ++ | + | - |
| 70℃, 10 min | + | - | - |
| 56℃, 30 min | + | - | - |
| 90℃, 10 min | - | - | - |

[ ++ means OCIF activity inhibiting osteoclast development more than 80%, +means OCIF activity inhibiting osteoclast development between 30% and 80%, and - means no OCIF activity.]

EXAMPLE 6

Internal amino acid sequence of OCIF protein

Each 2 fractions (1 ml) from [No. 51-70 of blue-5PW fraction was] acidified with 10 μl of 25% TFA, and [was] applied to a reverse phase C4 column (BU-300, 2.1x220mm, manufactured by Perkin-Elmer Co.) equilibrated with 25% [of] acetonitrile containing 0.1 % TFA. The adsorbed protein was eluted with a 12 ml linear gradient of 25 to 55% acetonitrile at a flow rate of 0.2 ml/min, and the protein fractions corresponding to [peak 6 and peak] 7 were collected, respectively. The protein [of] each peak was applied to a protein sequencer (PROCISE 494, Perkin-Elmer Co.). However, the N-terminal sequence of the [protein] of each peak could not be analyzed. Therefore, [N-terminal] of the protein of each peak was considered to be blocked. [So, internal] amino acid sequences of these proteins were analyzed.

The protein [of] peak 6 or [peak 7] purified by C4-HPLC, was concentrated by centrifugation and pyridilethylated under reducing conditions. Briefly, 50 μl of 0.5 M Tris-HCl, pH 8.5, containing 100 μg of dithiothreitol, 10mM EDTA, 7 M guanidine-HCl, and 1% CHAPS was added to each samples, and the [mixture was] incubated overnight in the dark at [a] room temperature. Each [the] mixture was acidified with 25% TFA (a final concentration 0.1%) and [was] applied to a [reversed] phase C4 column (BU-300, 2.1x30mm, Perkin-Elmer Co.) equilibrated with 20 % acetonitrile containing 0.1 % TFA. The pyridil-ethylated OCIF

protein was eluted with a 9 ml linear gradient from 20 to 50% acetonitrile at a flow rate of 0.3 ml/min, and each protein peak was collected. The pyridil-ethyrated OCIF protein was concentrated under vacuum and dissolved in 25 μl of 0.1 M Tris-HCl, pH 9, containing 8 M Urea, and 0.1 % Tween 80. Seventy three μl of 0.1 M Tris-HCl, pH 9, and 0.02 μg of lysyl endopeptidase (Wako Pure Chemical, Japan) were added to the tube, and incubated at 37 ℃ for 15 hours. Each digest was acidified with 1 μl of 25% TFA and was applied to a reverse phase C8 column (RP-300, 2.1x220mm, Perkin-Elmer Co.) equilibrated with 0.1% TFA.

The peptide fragments were eluted from the column with linear gradient from 0 to 50 % acetonitrile at a flow rate of 0.2 ml/min for 70 min., and each peptide peak was collected. Each peptide fragment (P1 - P3) was applied to the protein sequencer. The sequences of the peptides were shown in Sequence Numbers 1 - 3, respectively.

EXAMPLE 7

Determination of nucleotide sequence of the OCIF cDNA

i) Isolation of poly(A) + RNA from IMR-90 cells

About 10 ug of poly(A) + RNA was isolated from 1x10$^8$ cells of IMR-90 by using Fast Track mRNA isolation kit (Invitrogen) according to the manufacturer's instructions.

ii) Preparation of mixed primers

The following two mixed primers were synthesized based on the amino acid

sequences of two peptides (peptide P2 and peptide P3, [sequence numbers] 2 and 3, respectively). All the oligonucleotides in the mixed primers No. 2F can code for the amino acid sequence from the sixth residue, glutamine (Gln) to the twelfth residue, leucine (Leu), in peptide P2. All the oligonucleotides in the mixed primers No. 3R can code for the amino acid sequence from the sixth residue, histidine (His), to the twelfth residue, lysine (Lys), in peptide P3. The sequences of the mixed primers No. 2F and No. 3R were shown in Table 3.

Table 3

No. 2F

5'-CAAGAACAAA CTTTTCAATT-3'

G G G C C GC

A

G

No. 3R

5'-TTTATACATT GTAAAAGAAT G-3'

C G C G GCTG

A C

G T

iii) Amplification of OCIF cDNA fragment by PCR (Polymerase chain reaction)

First strand cDNA was generated using Superscript II cDNA synthesis kit

(Gibco BRL) and 1 [ug] of poly(A) + RNA obtained in the example 7-i), according to the manufacturer's instructions. The DNA fragment encoding OCIF was obtained by PCR using [the] cDNA template and the primers shown in EXAMPLE 7-ii).

PCR was performed [with the conditions as follows:]

| | |
|---|---|
| 10X Ex Taq Buffer (Takara Shuzo) | 5 [ul] |
| 2.5 mM solution of dNTPs | 4 [ul] |
| cDNA solution | 1 [ul] |
| Ex Taq (Takara Shuzo) | 0.25 [ul] |
| sterile distilled water | 29.75 [ul] |
| 40 [uM] solution of primers No. 2F | 5 [ul] |
| 40 [uM] solution of primers No. 3R | 5 [ul] |

The components of the reaction were mixed in a microcentrifuge tube. An initial denaturation step at 95 ℃ for 3 min was followed by 30 cycles of denaturation at 95℃ for 30 [sec annealing] at 50 ℃ for 30 sec and extention at 70 ℃ for 2min. After the amplification, [a] final extention step was performed at 70 ℃ for 5min. The [size of] PCR products were determined on a 1.5 % agarose gel electrophoresis. [About] 400 bp OCIF DNA fragment was obtained.

EXAMPLE 8

Cloning of the OCIF cDNA fragment amplified by PCR and determination of its DNA sequence

The OCIF cDNA fragment amplified by PCR in EXAMPLE 7-iii) was inserted in the plasmid, pBluescript II SK- using DNA ligation kit ver. 2 (Takara Shuzo) according to the method by Marchuk, D. et al. (Nucleic Acids Res., vol 19, p1154, 1991). E. coli. DH5 α (Gibco BRL) was transformed with ligation mixture. The transformants were grown and a plasmid containing the OCIF cDNA (about 400 bp) was purified using the commonly used method. This plasmid was called pBSOCIF. The sequence of OCIF cDNA in pBSOCIF was determined using Taq Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer). The size of the OCIF cDNA is 397 bp. The OCIF cDNA encodes an amino acid sequence containing 132 residues. The amino acid sequences of the internal peptides (peptide P2 and peptide P3, sequence number 2 and 3, respectively) that were used to design the primers were found at N- or C- terminal side in the amino acid sequence of the 132 amino acid polypeptide predicted by the 397 bp OCIF cDNA. In addition, the amino acid sequence of the internal peptide P1 (sequence number 1) was also found in the predicted amino acid sequence of the polypeptide. These data show that the 397 bp OCIF cDNA is a portion of the full length OCIF cDNA.

EXAMPLE 9

Preparation of the DNA probe

The 397 bp OCIF cDNA was prepared according to the conditions described in EXAMPLE 7-iii). The OCIF cDNA was subjected to a preparative agarose gel electrophoresis. The OCIF cDNA was purified from the gel using QIAEX gel extraction kit (QIAGEN), labeled with [$\alpha^{32}$P]dCTP using Megaprime DNA labeling

system (Amersham) and used to select a phage containing the full length OCIF cDNA.

EXAMPLE 10

Preparation of the cDNA library

cDNA was generated using a Great Lengths cDNA synthesis kit (Clontech), oligo (dT) primer, [$\alpha^{32}$P]dCTP and 2.5 µg of poly(A) + RNA obtained in the example 7-i), according to the manufacturer's instructions. An EcoRI-SalI-NotI adaptor was ligated to the cDNA. The cDNA was separated from the free adaptor and unincorporated free [$\alpha^{32}$P]dCTP. The purified cDNA was precipitated with ethanol and dissolved in 10 ul of TE buffer (10 mMTris-HCl (pH8.0), 1 mM EDTA). The cDNA comprising the adaptor was ligated into with the adaptor was inserted in λZAP EXPRESS vector (Stratagene) at the EcoRI site. The recombinant λZAP EXPRESS phage DNA containing the cDNA was in vitro packaged using a Gigapack gold II packaging extract (Stratagene) and yielding a recombinant λZAP EXPRESS phage library was prepared.

EXAMPLE 11

Screening of recombinant phage

Recombinant phages obtained in EXAMPLE 10 were used to infect E. coli strain infected to E. Coli, XL1-Blue MRF' (Stratagene) at 37 ℃ for 15 min.. The infected E. coli cells were added to NZY medium containing 0.7 % agar at 50℃ and plated onto the NZY agar plates. After the plates were incubated at 37 ℃ overnight, Hybond N membranes (Amersham) were placed on the surface of the plates containing plaques. The membranes were denatured in the alkali solution, neutralized, and washed in

2xSSC according to the standard protocol. The phage DNA was immobilized on the membranes using UV Crosslink (Stratagene). The membranes were incubated in the hybridization buffer (Amersham) containing 100 μg/ml salmon sperm DNA at 65℃ for 4 hours and then incubated at 65 ℃ overnight in the same buffer containing $2x10^5$ cpm/ml denatured OCIF DNA probe. The membranes were washed twice with 2xSSC and twice with a solution containing 0.1xSSC and 0.1 % SDS at 65 ℃ for 10 min each time. The positive clones were purified by repeating the screening twice. The purified λ ZAP EXPRESS phage clone containing about 1.6 kb DNA insert was used in the experiments described below. This phage was called λ OCIF. The purified λ OCIF and the infected into E. Coli XL1-Blue MRF' (Stratagene) according to the protocol of λ ZAP EXPRESS cloning kit (Stratagene). The culture broth of infected XL1-Blue MRF' was prepared. Purified λOCIF and ExAssist helper phage (Stratagene) were co-infected into E. coli strain XL-1 blue MRF' according to the protocol supplied with the kit. The culture broth of the co-infected XL-1 blue MRF' was added to a culture of E. coli strain XLOR (Stratagene) to transform them. Thus we obtained a Kanamycin-resistant transformant harboring a plasmid designated pBKOCIF which is a pBKCMV (Stratagene) vector containing the 1.6 kb insert fragment.

The transformant including the plasmid containing about 1.6 kb OCIF cDNA was obtained by picking up the kanamycin-resistant colonies. The plasmid was called pBKOCIF. The transformant has been deposited to National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Tecnology as "FERM BP-5267" as pBK/O1F10. A national deposit (Accession number, FERM P-14998) was transfered to the international deposit, on October 25, 1995

according to the Budapest treaty. The transformant pBK/01F10 was grown and the plasmid pBKOCIF was purified according to the standard protocol. standard methods.

EXAMPLE 12

Determination of the nucleotide sequence of OCIF cDNA containing the full coding region.

The nucleotide sequence of OCIF cDNA obtained in EXAMPLE 11 was determined using a Taq Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer). The primers used were T3, T7 primers (Stratagene) and synthetic primers designed according to the OCIF cDNA sequence. The sequences of these primers are shown in SEQ. ID Nos. sequence numbers 16 to 29. The nucleotide sequence of the OCIF cDNA is shown in SEQ. ID No. sequence number 6 and the amino acid sequence predicted by the cDNA sequence is shown in SEQ. ID No. sequence number 5.

EXAMPLE 13

Production of recombinant OCIF by 293/EBNA cells

i) Construction of the plasmid for expressing OCIF cDNA

pBKOCIF, containing about 1.6 kb OCIF cDNA, was prepared as described in EXAMPLE 1 and digested with restriction enzymes BamHI and XhoI. The OCIF cDNA insert was cut out, separated by an agarose gel electrophoresis and purified using a QIAEX gel extraction kit (QIAGEN). The purified OCIF cDNA insert was ligated using DNA ligation kit ver. 2 (Takara Shuzo) to the insert was ligated into the using DNA ligation Kit ver. 2 (Takara Shuzo) expression vector pCEP4 (Invitrogen) digested with restriction enzymes BamHI and XhoI. E. coli. E. Coli strain DH5α (Gibco BRL) was transformed with the ligation mixture.

The transformants were grown and the plasmid containing the OCIF cDNA (about 1.6 kb) was purified using a QIAGEN column (QIAGEN). The expression plasmid pCEPOCIF was precipitated with ethanol and dissolved in sterile distilled water for use in the experiments.

ii) Transient expression of OCIF cDNA and analysis of the biological activity

Recombinant OCIF was produced using the expression plasmid pCEPOCIF (prepared in EXAMPLE 13-i) according to the method described below. $8 \times 10^5$ cells of 293/EBNA (Invitrogen) were inoculated into each well of a 6-well plate using IMDM containing 10 % fetal calf serum (Gibco BRL). After the cells were incubated for 24 hours, the culture medium was removed and the cells were washed with serum free IMDM. The expression plasmid pCEPOCIF and lipofectamine (Gibco BRL) were diluted with OPTI-MEM (Gibco BRL), and were mixed, and added to the cells in each well according to the manufacture's instructions. Three $\mu$g of pCEPOCIF and 12 $\mu$l of lipofectamine were used for each transfection. After the cells were incubated with pCEPOCIF and lipofectamine for 38 hours, the medium was replaced with 1 ml of OPTI-MEM. After the transfected cells were incubated for 30 hours, the conditioned medium was harvested and used for the biological assay. The biological activity of OCIF was analysed according to the method described below. Bone marrow cells obtained from mice, 17 days-old, were suspended in $\alpha$-MEM (manufactured by GIBCO BRL Co.) containing 10% FBS, $2 \times 10^{-8}$M activated vitamin $D_3$ and each test sample, and were inoculatd and cultured for 7 days at 37℃ in humidified 5%$CO_2$ as described in EXAMPLE 2. During incubation, 160

$\mu$l of old medium in each well was replaced with the same volume of the fresh medium containing test sample diluted with $1x10^{-8}$M of activated vitamin $D_3$ and $\alpha$-MEM containing FBS on day 3 and day 5. On day 7, after washing the wells with phosphate buffered saline, cells were fixed with ethanol/acetone (1:1) for 1 min. and then osteoclast development was tested using an acid phosphatase activity mesuring kit (Acid Phosphatase, Leucocyte, Catalog No. 387-A, Sigma Co.). A decrease in the number of TRAP positive cells was taken as an OCIF activity. The conditioned medium showed the same OCIF activity as natural OCIF protein from IMR-90 conditioned medium (Table 4).

Table 4

OCIF activity of 293/EBNA conditioned medium.

| Cultured Cell | Dilution | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 |
| OCIF expression vector transfected | ++ | ++ | ++ | ++ | ++ | + | - |
| vector transfected | - | - | - | - | - | - | - |
| untreated | - | - | - | - | - | - | - |

[ ++ ; OCIF activity inhibiting osteoclast development more than 80%, + ; OCIF activity inhibiting osteoclast development between 30% and 80%, and - ; no

OCIF activity. ]

iii) Isolation of recombinant OCIF protein from 293/EBNA-conditioned medium

293/EBNA-conditioned medium (1.8 l) obtained by cultivating the cells described in example 13-ii) was supplemented with 0.1 % of CHAPS and filtrated using a with 0.22 μm membrane filter (Steribecs GS, Millipore Milipore Co.). The conditioned medium was applied to a 50 ml of heparin Sepharose CL-6B column (2.6 x 10 cm, Pharmacia Co.) equilibrated with 10mM Tris-HCl, pH 7.5. After washing the column with 10mM Tris-HCl, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 4 ml/min for 100 min. and 8 ml fractions (8 ml) were collected. Using 150 μl of each fraction, OCIF activity was assayed according to the method described in EXAMPLE 2. An OCIF active 112 ml fraction OCIF active fraction (112 ml) eluted with approximately 0.6 to 1.2 M NaCl, was obtained.

One hundred twelve ml of the active fraction was diluted to 1000 ml with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5, and applied to a heparin affinity column (heparin-5PW, 0.8 x 7.5 cm, Tosoh Co.) equilibrated with 10mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 10mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 0.5ml/min for 60 min. and 0.5 ml fractions (0.5 ml) were collected. Four μl of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described in EXAMPLE 4. On SDS-PAGE under reducing conditions, A single band of rOCIF protein with an apparent 60 kD was detected in fractions from 30 to 32 by SDS-PAGE under reducing conditions.

non-reducing conditions, bands of rOCIF protein with an apparent 60 KD and 120 KD were also detected in fractions from 30 to 32. The isolated rOCIF fraction from 30 to 32 was designated as recombinant OCIF derived from 293/EBNA (rOCIF(E)). 1.5 ml of the rOCIF(E) (535 μg/ml) was obtained when determined by the method of Lowry, using bovine serum albumin as a standard protein.

EXAMPLE 14

Production of recombinant OCIF using CHO cells

i) Construction of the plasmid for expressing OCIF

pBKOCIF containing about 1.6 kb OCIF cDNA was prepared as described in EXAMPLE 11, and digested with restriction enzymes, SalI and EcoRV. About 1.4 kb OCIF cDNA insert was separated by an agarose gel electrophoresis, and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The expression vector, pcDL-SR α296 (Molecular and Cellular Biology, vol 8, p466, 1988) was digested with restriction enzymes, PstI and KpnI. About 3.4 kb of the expression vector fragment was cut out, separated by agarose gel electrophoresis, and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The ends of the purified OCIF cDNA insert and the expression vector fragment were blunted using a DNA blunting kit (Takara Shuzo). The purified OCIF cDNA insert and the expression vector fragment were ligated using a DNA ligation kit ver. 2 (Takara Shuzo). E. coli strain DH5a α (Gibco BRL) was transformed with the ligation mixture. A transformant containing the OCIF expression plasmid, pSRαOCIF was obtained.

ii) Preparation of the expression plasmid

The transformant containing the OCIF expression plasmid, pSR αOCIF preprared in the example 13-i) and the transformant containing the mouse DHFR expression plasmid, pBAdDSV shown in WO92/01053 were grown according to the standard method. Both plasmids were purified by alkali treatment, polyethylene glycol precipitation, and cesium chrolide density gradient ultra centrifugation according to the method of Maniatis et al. (Molecular cloning, 2nd edition).

iii) Adaptation of CHOdhFr- cells to the protein free medium

CHOdhFr- cells (ATCC, CRL 9096) were cultured in IMDM containing 10 % fetal calf serum. The cells were adapted to EX-CELL 301 (JRH Biosciecnce) and then adapted to EX-CELL PF CHO (JRH Biosciecnce) according to the manufacture's instructions.

iv) Transfection of the OCIF expression plasmid, and the mouse DHFR expression plasmid, to CHOdhFr- cells.

CHOdhFr- cells prepared in EXAMPLE 14-iii) were transfected by electroporation with pSRαOCIF and pBAdDSV prepared in EXAMPLE 14-ii). 200 μg of pSRαOCIF and 20 μg of pBAdDSV were dissolved under sterile conditions in 0.8 ml of IMDM (Gibco BRL) containing 10 % fetal calf serum. $2 \times 10^7$ cells of CHOdhFr- were suspended in 0.8 ml of this medium. The cell suspension was transfered to a cuvette (Bio Rad) and the cells were transfected by electroporation using a gene pulser (Bio Rad) under condition of

360 V and 960 μF. The suspension of electroporated cells was transferred to T-flasks (Sumitomo Bakelite) containing 10 ml of EX-CELL PF-CHO, and incubated in the $CO_2$ incubator for 2 days. Then the transfected cells were inoculated in each well of a 96 well plate (Sumitomo Bakelite) at a density of 5000 cells/well and cultured for about 2 weeks. The transformants expressing DHFR are selected since EX-CELL PF-CHO does not contain nucleotides and the parental cell line CHO dhFr- can not grow in this medium. Most of the transformants expressing DHFR express OCIF since the OCIF expression plasmid was used ten times as much as the mouse DHFR expression plasmid. The transformants whose conditioned medium had high OCIF activity were selected among the transformants expressing DHFR according to the method described in EXAMPLE 2. The transformants that express large amounts of OCIF were cloned by limiting dilution. The clones whose conditioned medium had high OCIF activity were selected as described above and the transformant expressing large amounts of OCIF, named 5561, was obtained.

v) Production of recombinant OCIF

To produce recombinant OCIF (rOCIF), EX-CELL 301 medium (3 l) in a 3 l-spiner flask was inoculated with the clone (5561) at a cell-density of $1x10^5$ cells/ml. The 5561 cells were cultured in a spiner flask at 37℃ for 4 to 5 days. When the concentration of the 5561 cells reached to $1x10^6$ cells/ml, about 2.7 l of the conditioned medium was harvested. Then about 2.7 l of EX-CELL 301 was added to the spiner flask and the 5561 cells were cultured repeatedly. About 20 l of the conditioned medium was harvested using the three spiner

flasks.

vi) Isolation of recombinant OCIF protein from CHO cells-conditioned medium (CHO cell-conditioned medium (1.0 l) described in EXAMPLE 14-v) was supplemented with 1.0 g of CHAPS and filtrated with a 0.22 μm membrane filter (Steribecks GS, Millipore Co.). The conditioned medium was applied to a heparin Sepharose-FF column (2.6 x 10 cm, Pharmacia Co.) equilibrated with 10 mM Tris-HCl, pH 7.5. After washing the column with 10 mM Tris-HCl, 0.1 % CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 4 ml/min for 100 min. and 8ml fractions (8 ml) were collected. Using 150 μl of each fraction, OCIF activity was assayed according to the method described in EXAMPLE 2. An active fraction (112 ml) eluted with approximately 0.6 to 1.2 M NaCl was obtained.

The 112 ml of active fraction was diluted to 1200 ml with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5, and applied to an affinity column (Blue-5PW, 0.5 x 5.0 cm, Tosoh Co.) equilibrated with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5. After washing the column with 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 3 M NaCl at a flow rate of 0.5ml/min for 60 min., and fractions (0.5 ml) were collected. Four μl of each fraction were subjected to SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described in EXAMPLE 4. On SDS-PAGE under reducing conditions, a single band of rOCIF protein with an apparent molecular weight of 60 KD was detected in fractions 30 to 38 using SDS-PAGE under reducing conditions. Bands under non-reducing conditions, bands of rOCIF protein with apparent molecular weights of 60 KD and 120 KD using SDS-PAGE under non-reducing conditions were also detected in fractions

from fractions

30 to 38. The isolated rOCIF fraction, 30 to 38, was designated as purified recombinant OCIF derived from CHO cells (rOCIF(C)). 4.5 ml of the rOCIF(C) (113 $\mu$g/ml) was obtained, as obtained when determined by the method of Lowry using bovine serum albumin as a standard protein.

EXAMPLE 15

Determination of N-terminal amino acid sequence of rOCIFs

Each 3 μg of the isolated rOCIF(E) and rOCIF(C) was adsorbed to polyvinylidene difluoride (PVDF) membranes with Prospin (PERKIN ELMER Co.). The membranes were washed with 20 % ethanol and the N-terminal amino acid sequences of the adsorbed proteins were analyzed by protein sequencer (PROCISE 492, PERKIN ELMER Co.). The determined N-terminal amino acid sequence is shown in [sequence No.] ^SEQ. ID No. 7.

The N-terminal amino acid of rOCIF(E) and rOCIF(C) was [the 22th amino acid of glutamine from Met as translation starting point, as shown in sequence number] ^glutamic acid located at position 22 ^from Met of the translation start site, as shown in SEQ. ID No. 5. The 21 amino acids from Met to Gln were identified as a signal peptide. The N-terminal amino acid sequence of OCIF isolated from IMR-90 conditioned medium [was undetectable.] ^could not be determined. Accordingly, the N-terminal [glutamine] ^glutamic acid of OCIF may be blocked by [converting from glutamine] ^the conversion of glutamic acid to pyroglutamine within [culturing or purifing.] ^cell culture or purification steps.

EXAMPLE 16

Biological activity of recombinant(r) OCIF and natural(n) OCIF

i) Inhibition of vitamin $D_3$ induced osteoclast formation [from] ^in murine bone marrow cells

Each the rOCIF(E) and nOCIF [sample was] ^samples were diluted with α-MEM (GIBCO BRL Co.) containing 10% FBS and $2 \times 10^{-8}$M of activated vitamin $D_3$ (a final concentration of 250 ng/ml). Each sample was serially diluted with the same medium, and 100 μl of each diluted sample was added to each well [in] ^of a 96-well

plates. Bone marrow cells obtained from mice, 17 days-old, were inoculated at a cell density of $3x10^5$ cells/100 μl/ well to each well in 96-well plates and cultured for 7 days at 37℃ in humidified 5%$CO_2$. On day 7, the cells were fixed and stained with a acid phosphatase mesuring kit (Acid Phosphatase, Leucocyte, No387-A, Sigma) according to the method described in EXAMPLE 2. The decrease of acid phosphatase activity (TRAP) was taken as OCIF activity. The decrease of acid phosphatase-positive cells was evaluated by solubilizing the pigment of dye and measuring absorbance. In detail, 100 μl of a mixture of 0.1 N NaOH and dimethylsulfoxide (1:1) was added to each well and the well was vibrated to solubilize the dye. After solubilizing the dye completely, an absorbance of each well was measured at 590 nm, subtracting the absorbance at 490 nm using a microplate reader (Immunoreader NJ-2000, InterMed). The microplate reader was adjusted to 0 absorbance using a well with monolayered bone marrow cells which was cultured in the medium without activated vitamin $D_3$. The decrease of TRAP activity was expressed as a percentage of the control absorbance value (=100%) of the solubilized dye from wells with bone marrow cells which were cultured in the absence of OCIF. The results are shown in Table 5.

Table 5

Inhibition of vitamin D3-induced osteoclast formation from murine bone marrow cells

| OCIF concentration(ng/ml) | 250 | 125 | 63 | 31 | 16 | 0 |
|---|---|---|---|---|---|---|
| rOCIF(E) | 0 | 0 | 3 | 62 | 80 | 100 |

| | | | | | | |
|---|---|---|---|---|---|---|
| nOCIF | 0 | 0 | 27 | 27 | 75 | 100 (%) |

Both nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner [in the concentration] of 16 ng/ml or [higher] ^ greater.

ii) Inhibition of vitamin D3-induced osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

[Effect] ^ The effect of OCIF on osteoclast formation induced by Vitamin $D_3$ in co-cultures of stromal cells and mouse spleen cells was tested according to the method of N. Udagawa et al. (Endocrinology, vol. 125, p1805-1813, 1989). [In detail,] ^ Briefly, samples of each of rOCIF(E), rOCIF(C), and nOCIF [sample was] ^ were serially diluted with $\alpha$-MEM (GIBCO BRL Co.) containing 10% FBS, $2\times10^{-8}$M [of] activated vitamin $D_3$, and $2\times10^{-7}$M dexamethasone [,] and 100 $\mu$l of each ^ of the diluted samples was added to each well [in] ^ of 96 well-microwell plates. Murine bone marrow-derived stromal ST2 cells (RIKEN Cell Bank RCB0224) [;] ^ at $5\times10^3$ cells per 100 $\mu$l of $\alpha$-MEM containing 10% [FBS,] ^ FBS and spleen cells from [ddy mice, 8 weeks-old, ;] ^ 8 week old ddy mice at $1\times10^5$ cells per 100 $\mu$l in the same medium, were inoculated [to] ^ into each well [in 96-well plates] ^ of a 96-well plate and cultured for 5 days at 37°C in humidified 5%$CO_2$. On day 5, the cells were fixed and stained [with a kit for] ^ using an acid phosphatase ^ kit (Acid Phosphatase, Leucocyte, No387-A, Sigma). [The decrease of] ^ A decrease in acid phosphatase-positive cells was taken ^ as an indication as OCIF activity. The decrease [of] ^ in acid phosphatase-positive cells was evaluated according to the method described in EXAMPLE 16-i). The results [are] ^ are shown in Table 6 (rOCIF(E) and rOCIF(C)) and Table 7 (rOCIF(E) and [nOCIF.] ^ nOCIF).

Table 6

Inhibition of osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

| OCIF concentration(ng/ml) | 50 | 25 | 13 | 6 | 0 | |
|---|---|---|---|---|---|---|
| rOCIF(E) | 3 | 22 | 83 | 80 | 100 | |
| rOCIF(C) | 13 | 19 | 70 | 96 | 100 | (%) |

Table 7

Inhibition of osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

| OCIF concentration(ng/ml) | 250 | 63 | 16 | 0 | |
|---|---|---|---|---|---|
| rOCIF(E) | 7 | 27 | 37 | 100 | |
| rOCIF(C) | 13 | 23 | 40 | 100 | (%) |

nOCIF, rOCIF(E) and rOCIF(C) inhibited osteoclast formation in a dose dependent manner [in ^at concentrations the concentration] of 6 - 16 ng/ml or [higher] → greater.

iii) Inhibition of PTH-induced osteoclast formation [from] ^in murine bone marrow cells.

^The effect

[Effect] of OCIF on osteoclast formation induced by PTH was tested according to the method of N. Takahashi et al. (Endocrinology, vol. 122,

p1373-1382, 1988). Briefly, samples of each of In detail, each the rOCIF(E) and nOCIF sample (125 ng/ml) was were serially diluted with α-MEM (manufactured by GIBCO BRL Co.) containing 10% FBS and $2x10^{-8}$M PTH, and 100μl of each of the diluted samples was added to the wells of 96 well-plates. Bone marrow cells from ddy mice, 17 days-old, 17 day old ddy mice at a cell density of $3x10^5$ cells per 100μl of α-MEM containing 10% FBS were inoculated to into each well in of a 96-wells plates plate and cultured for 5 days at 37℃ in humidified 5%$CO_2$. On day 5, the cells were fixed with ethanol/aceton acetone (1:1) for 1 min. at room temperature and stained with a kit for an acid phosphatase Kit (Acid Phosphatase, Leucocyte, No387-A, Sigma) according to the method described in EXAMPLE 2. The A decrease of in acid phosphatase-positive cells was taken as an indication of OCIF activity. The decrease of in acid phosphatase-positive cells was evaluated according to the method described in EXAMPLE 16-i). The results are shown in Table 8.

Table 8

Inhibition of PTH-induced osteoclast formation from murine bone marrow cells.

| OCIF concentration(ng/ml) | 125 | 63 | 31 | 16 | 8 | 0 |
|---|---|---|---|---|---|---|
| rOCIF(E) | 6 | 58 | 58 | 53 | 88 | 100 |
| nOCIF | 18 | 47 | 53 | 56 | 91 | 100 |

nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner in the at concentrations concentration of 16 ng/ml or higher greater.

iv) Inhibition of IL-11-induced osteoclast formation

Effect of OCIF on osteoclast formation induced by IL-11 was tested according to the method of T. Tamura et al. (Proc. Natl. Acad. Sci. USA, vol. 90, p11924-11928, 1993). In detail, each rOCIF(E) and nOCIF sample was serially diluted with α-MEM (GIBCO BRL Co.) containing 10% FBS and 20 ng/ml IL-11 and 100μl of each the diluted sample was added to each well in 96-well plates. Newborn mouse calvaria-derived pre-adipocyte MC3T3-G2/PA6 cells (RIKEN Cell Bank RCB1127) ; $5x10^3$ cells per 100μl of α-MEM containing 10% FBS, and spleen cells from ddy mouse, 8 weeks-old, ; $1x10^5$ cells per 100 μl in the same medium, were inoculated to each well in 96-well plates and cultured for 5 days at 37 ℃ in humidified 5%$CO_2$. On day 5, the cells were fixed and stained with a kit for acid phosphatase (Acid Phosphatase, Leucocyte, No387-A, Sigma). Acid phosphatase positive cells were counted under microscope and a decrease of the cell numbers was taken as OCIF activity. The results are shown in Table 9.

Table 9

| OCIF concentration(ng/ml) | 500 | 125 | 31 | 7.8 | 2.0 | 0.5 | 0 |
|---|---|---|---|---|---|---|---|
| nOCIF | 0 | 0 | 1 | 4 | 13 | 49 | 31 |
| rOCIF(E) | 0 | 0 | 1 | 3 | 10 | 37 | 31 |

Both nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner in the concentration of 2 ng/ml or higher.

The results shown in Table 4-8 indicated that OCIF inhibits all the

vitamin $D_3$, PTH, and IL-11-induced osteoclast formations at almost the same doses. Accordingly, OCIF [would ^could be able to] be used for [treatment of the] ^treating different types of bone disorders [with] ^due to decreased bone mass, [which] ^that are caused by different substances [which] ^that induce bone resorption.

EXAMPLE 17

Isolation of monomer-type OCIF and dimer-type OCIF

Each rOCIF(E) and rOCIF(C) sample containing 100 μg of OCIF protein, was supplemented with 1/100 volume of 25 % trifluoro acetic acid and applied to a reverse phase column (PROTEIN-RP, 2.0x250 mm, YMC Co.) equilibrated with 30 % acetonitrile containing 0.1 % trifluoro acetic acid. OCIF protein was eluted from the column with ^a linear gradient from 30 to 55 % acetonitrile at a flow rate of 0.2 ml/min for 50 min. and each OCIF peak was collected. [Each the] ^The monomer-type OCIF peak fraction and dimer-type OCIF peak fraction [was] ^were [lyophilized, respectively.] ^each lyophilized.

EXAMPLE 18

Determination of ^the molecular weight of recombinant OCIFs

Each 1 μg of the isolated monomer-type and dimer-type nOCIF purified using ^a reverse phase column according to EXAMPLE 3-iv) and each 1 μg of monomer-type and dimer-type rOCIF described in EXAMPLE 17 was concentrated under [vaccum, respectively] ^vacuum. Each sample was incubated in the buffer for SDS-PAGE, subjected to SDS-polyacrylamide gel electrophoresis, and protein bands on the gel were stained with silver according to the method described in

EXAMPLE 4. Results of electrophoresis under non-reducing conditions and reducing conditions are shown in [Figure 6 and Figure 7.] ^ Figures 6 and 7, respectively.

A protein band with an apparent molecular weight of 60 KD was detected in each monomer-type OCIF sample, and a protein band with an apparent molecular weight of 120 KD was detected in each dimer-type OCIF sample [in] ^ under non-reducing conditions. A protein band with an apparent molecular weight of 60 KD was detected in each monomer-type OCIF sample under reducing conditions. Accordingly, ^ the molecular weights of monomer-type nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells and rOCIF from CHO cells were ^ almost the same (60KD). Molecular weights of dimer-type nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells, and rOCIF from CHO cells were also the same (120KD).

EXAMPLE 19

[Remove] ^ Removal of the N-linked Oligosaccharide chain and [Mesuring] ^ measuring the molecular weight of natural and recombinant OCIF

Each sample containing 5 $\mu$g of the isolated monomer-type and dimer-type nOCIF purified using ^ a reverse phase column according to EXAMPLE 3-iv) and each sample containing 5 $\mu$g of monomer-type and dimer-type rOCIF described in EXAMPLE 17 were concentrated under vaccum. Each sample was dissolved in 9.5 $\mu$l of 50 mM sodium phosphate buffer, pH 8.6, containing 100 mM 2-mercaptoethanol, supplemented with 0.5 $\mu$l of 250 U/ml N-glycanase (Seikagaku kogyo Co.) and incubated for one day at 37 ℃. Each sample was supplemented with 10 $\mu$l of 20 mM Tris-HCl, pH 8.0 containing 2 mM EDTA, 5 % SDS, and 0.02 % bromo-phenol blue and heated for 5 min at 100 ℃. Each 1 $\mu$l

of the samples was subjected to SDS-polyacrylamide gel electrophoresis [1] and protein bands on the gel were stained with silver as described in EXAMPLE 4. The patterns of electrophoresis are shown in Figure 8.

An apparent molecular weight of each of the deglycosylated nOCIF from IMR-90 cells, rOCIF from CHO cells, and rOCIF from 293/EBNA cells was 40 KD under reducing conditions. An apparent molecular weight of each of the untreated nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells, and rOCIF from CHO cells was 60 KD under reducing conditions. Accordingly, the results indicate that the OCIF proteins are glycoproteins with N-linked sugar chains.

EXAMPLE 20

Cloning of OCIF variant cDNAs and determination of their DNA [squences] ^ sequences

The plasmid pBKOCIF, [which ^ comprising is inserted] OCIF cDNA [to ^ into plasmid] pBKCMV (Stratagene), was obtained [from one of some purified positive phage] as in example 10 and 11. [And more, ^ Further during the screening of the cDNA library with the 397 bp OCIF cDNA probe, the transformants containing plasmids whose insert sizes were different from that of pBKOCIF were obtained. These transformants containing the plasmids were grown and the plasmids were purified according to the standard method. The sequence of the insert DNA in each plasmid was determined using ^ a Taq Dye Deoxy Terminater Cycle Sequencing kit (Perkin Elmer). The [used primers] ^ primers used were T3, T7, [primers] (Stratagene) and synthetic primers prepared based on the nucleotide sequence of OCIF cDNA. There are four OCIF variants (OCIF2, 3, 4, and 5) in addition to OCIF. The nucleotide sequence of OCIF2 is shown in [the] [sequence number ^ SEQ. ID No.] 8 and the amino acid sequence of OCIF 2 predicted by the nucleotide sequence is shown in [the sequence number ^ SEQ. ID No.] 9. The nucleotide sequence of OCIF3 is shown in [the sequence number ^ SEQ. ID No.] 10 and the amino acid sequence of OCIF3 predicted by the nucleotide sequence is shown in [the sequence number ^ SEQ. ID No.] 11. The nucleotide sequence of OCIF4 is shown in [the sequence number ^ SEQ. ID No.] 12 and the amino acid sequence of OCIF4 predicted by the nucleotide sequence is shown in [the sequence number ^ SEQ. ID No.] 13. The nucleotide sequence of OCIF5 is shown in [the] [sequence number ^ SEQ. ID No.] 14 and the amino acid sequence of OCIF5 predicted by the nucleotide sequence is shown in [the sequence number ^ SEQ. ID No.] 15. The structures of OCIF variants are shown in Figures 9 to 12 and are [described in brief ^ briefly] below.

OCIF2

The OCIF2 cDNA has a deletion of 21 bp from guanine at nucleotide number 265 to guanine at nucleotide number 285 in the OCIF cDNA (SEQ. ID No. 6). Accordingly, OCIF2 has a deletion of 7 amino acids from glutamic acid (Glu) at amino acid number 68 to glutamine (Gln) at amino acid number 74 in OCIF (SEQ. ID No. 5).

OCIF3

The OCIF3 cDNA has a point mutation at nucleotide number 9 in the OCIF cDNA (SEQ. ID No. 6) where cytidine is replaced with guanine. Accordingly, OCIF3 has a mutation and where asparagine (Asn) at amino acid number -19 in OCIF (SEQ. ID No. 5) is replaced with lysine (Lys). The mutation seems to be located in the signal sequence and has no essential effect on the secretion of secreted OCIF3. OCIF3 cDNA has a deletion of 117 bp from guanine at nucleotide number 872 to cytidine at nucleotide number 988 in the OCIF cDNA (SEQ. ID No. 6).

Accordingly, OCIF3 has a deletion of 39 amino acids from threonine (Thr) at amino acid number 270 to leucine (Leu) at amino acid number 308 in OCIF (SEQ. ID No. 5).

OCIF4

The OCIF4 cDNA has two point mutations in the OCIF cDNA (SEQ. ID No. 6).

~~[OCIF4 cDNA has two point mutations in OCIF cDNA (sequence number 6).]~~

Cytidine at nucleotide number 9 is replaced with guanine and guanine at nucleotide number 22 is replaced with thymidine in the OCIF cDNA (SEQ. ID No. 6). Accordingly, OCIF4 has two mutations. Asparagine (Asn) at amino acid number -19 in OCIF (SEQ. ID No. 5) is replaced with lysine (Lys), and alanine (Ala)

at amino acid number -14 is replaced with serine (Ser). These mutations seem to be located in the signal sequence and have no essential effect on the secreted OCIF4.

OCIF4 cDNA has about 4 kb DNA, [which is the] intron 2 of OCIF gene, inserted between nucleotide number 400 and nucleotide number 401 in OCIF cDNA ([sequence number] 6). The open reading frame stops in intron 2.

Accordingly, OCIF4 has an additional novel amino acid sequence containing 21 amino acids after alanine (Ala) at amino acid number 112 in OCIF ([sequence number 5).]

OCIF5

OCIF5 cDNA has a point mutation at nucleotide number 9 in OCIF cDNA ([sequence number] 6) where cytidine is replaced with guanine.

Accordingly, OCIF5 has a mutation [and] asparagine (Asn) at amino acid number -19 in OCIF ([sequence number] 5) is replaced with lysine (Lys). The mutation seems to be located in the signal sequence and [have] no essential effect on the [secreted] OCIF5.

OCIF5 cDNA has the latter portion (about 1.8 kb) of intron 2 between nucleotide number 400 and nucleotide number 401 in OCIF cDNA ([sequence number] 6). The open reading frame stops in the latter portion of intron 2.

Accordingly, OCIF5 has an additional novel amino acid sequence containing 12 amino acids after alanine (Ala) at amino acid number 112 in OCIF ([sequence number 5).]

EXAMPLE 21

Production of OCIF variants

i) Construction of the plasmid for expressing OCIF variants

The plasmid containing OCIF2 or OCIF3 cDNA was obtained as described in EXAMPLE 20 and called pBKOCIF2 and pBKOCIF3, respectively. pBKOCIF2 and pBKOCIF3 were digested with restriction enzymes, BamHI and XhoI. The OCIF2 and OCIF3 cDNA inserts were separated by agarose gel electrophoresis, and purified from the gel using QIAEX gel extraction kit (QIAGEN). The purified OCIF2 and OCIF3 cDNA inserts were individually ligated using DNA ligation kit ver. 2 (Takara Shuzo) to the expression vector pCEP4 (Invitrogen) that had been digested with restriction enzymes, BamHI and XhoI. E.coli DH5 α (Gibco BRL) was transformed with the ligation mixture.

The plasmid containing OCIF4 cDNA was obtained as described in EXAMPLE 20 and called pBKOCIF4. pBKOCIF4 was digested with restriction enzymes, SpeI and XhoI (Takara Shuzo). The OCIF4 cDNA insert was separated by an agarose gel electrophoresis, and purified from the gel using QIAEX gel extraction kit (QIAGEN). The purified OCIF4 cDNA insert was ligated using DNA ligation kit ver. 2 (Takara Shuzo) to the expression vector pCEP4 (Invitrogen) that had been digested with restriction enzymes, NheI and XhoI (Takara Shuzo). E. coli DH5 α (Gibco BRL) was transformed with the ligation mixture.

The plasmid containing OCIF5 cDNA was obtained as described in EXAMPLE 20 and was called pBKOCIF5. pBKOCIF5 was digested with restriction enzyme, HindIII (Takara Shuzo). The 5' portion of the coding region in the OCIF5 cDNA insert was separated by agarose gel electrophoresis, and purified from the gel using QIAEX gel extraction kit (QIAGEN). The OCIF expression plasmid, pCEPOCIF,

obtained in EXAMPLE 13-i) was digested with the restriction enzyme, HindIII (Takara Shuzo). The 5' portion of the coding region in the OCIF cDNA was removed. The rest of the plasmid that contains pCEP vector and the 3' portion of the coding region of OCIF cDNA was called pCEPOCIF-3'. pCEPOCIF-3' was separated by an agarose gel electrophoresis, and purified from the gel using a QIAEX gel extraction kit (QIAGEN). The OCIF5 cDNA HindIII fragment and pCEPOCIF-3' were ligated using a DNA ligation kit ver. 2 (Takara Shuzo). E. coli strain coli. DH5 $\alpha$ (Gibco BRL) was transformed with the ligation mixture.

The transformants obtained were grown at 37 ℃ overnight and the OCIF variant variants expression plasmids (pCEPOCIF2, pCEPOCIF3, pCEPOCIF4, and pCEPOCIF5) were purified using QIAGEN column columns (QIAGEN). These OCIF variant variants expression plasmids were precipitated with ethanol, dissolved in sterile distilled water, and used in the experiments described below.

ii) Transient expression of OCIF variant cDNAs and analysis of the biological activity of recombinant OCIF variants.

Recombinant OCIF variants were produced using the expression plasmids, plasmid pCEPOCIF2, pCEPOCIF3, pCEPOCIF4, and pCEPOCIF5 prepared as described in EXAMPLE 21-i) according to the method described in EXAMPLE 13-ii). The biological activities of recombinant OCIF variants were analysed. The results were that these OCIF variants (OCIF2, OCIF3, OCIF4, and OCIF5) had a weak activity.

EXAMPLE 22

Preparation of OCIF mutants

i) Construction of a plasmid vector for subcloning cDNAs encoding OCIF mutants

The plasmid vector (5 μg) described in EXAMPLE 11 was digested with restriction enzymes Bam HI and Xho I ( Takara Shuzo). The digested DNA was subjected to preparative agarose gel electrophoresis. A DNA fragment with an approximate size of 1.6 kilobase pairs (kb) that contained the entire coding sequence for OCIF was purified from the gel using a QIAEX gel extraction kit (QIAGEN). The purified DNA was dissolved in 20 μl of sterile distilled water. This solution was designated DNA solution 1. p Bluescript II SK + (3 μg) (Stratagene) was digested with restriction enzymes Bam HI and Xho I (Takara Shuzo). The digested DNA was subjected to preparative agarose gel electrophoresis. A DNA fragment with an approximate size of 3.0 kb was purified from the gel using a QIAEX DNA extraction kit (QIAGEN). The purified DNA was dissolved in 20 μl of sterile distilled water. This solution was designated DNA solution 2. One microliter of DNA solution 2, 4 μl of DNA solution 1 and 5 μl of ligation buffer I from a DNA ligation kit ver. 2 (Takara Shuzo) were mixed and incubated at 16 ℃ for 30 min. (The ligation mixture was used in the transformation of E. coli in a manner described below). Conditions for transformation of E. coli were as follows. One hundred microliters of competent E. coli strain DH5 α cells (GIBCO BRL) and 5 μl of the ligation mixture were mixed in a sterile 15-ml tube (IWAKI glass). The tube was kept on ice for 30 min. After incubation for 45 sec at 42℃, was added to the cells 250 μl of L broth (1% Tryptone, 0.5% yeast extract, 1% NaCl). The cell suspension was then incubated for 1hr. at 37℃ with shaking. Fifty

microliters of the cell suspension was plated onto an L-agar plate containing 50 μg/ml of ampicillin. The plate was incubated overnight at 37℃.

Six colonies which grew on the plate were individually incubated in 2 ml each of L-broth containing 50 μg/ml of ampicillin overnight at 37℃ with shaking. The structure of the plasmids in the colonies was analyzed. A plasmid in which the 1.6-kb DNA fragment containing the entire OCIF cDNA is inserted between the digestion sites of Bam HI and Xho I of pBluescript II SK + was obtained and designated as pSK + -OCIF.

ii) Preparation of mutants in which one of the Cys residues in OCIF is replaced with a Ser residue

1) Introduction of mutations into OCIF cDNA

OCIF mutants were prepared in which one of the five Cys residues present in OCIF at positions 174, 181, 256, 298 and 379 (in SEQUENCE NO 4) was replaced with a Ser residue and were designated OCIF-C19S(174Cys to Ser), OCIF-C20S (181Cys to Ser), OCIF-C21S (256Cys to Ser), OCIF-C22S (298Cys to Ser) and OCIF-C23S (379Cys to Ser), respectively.

To prepare the mutants, nucleotides encoding the corresponding Cys residues were replaced with those encoding Ser. Mutagenesis was carried out by a two-step polymerase chain reaction (PCR). The first step of the PCRs consisted of two reactions, PCR 1 and PCR 2.

| | | |
|---|---|---|
| PCR 1 | 10X Ex Taq Buffer (Takara Shuzo) | 10 μl |
| | 2.5 mM solution of dNTPs | 8 μl |
| | the plasmid vector described in EXAMPLE 11 (8ng/ml) | 2 μl |
| | sterile distilled water | 73.5 μl |

| | | | |
|---|---|---|---|
| | 20 $\mu$M solution of primer 1 | 5 | $\mu$l |
| | 100 $\mu$M solution of primer 2 (for mutagenesis) | 1 | $\mu$l |
| | Ex Taq (Takara Shuzo) | 0.5 | $\mu$l |
| PCR 2 | 10X Ex Taq Buffer (Takara Shuzo) | 10 | $\mu$l |
| | 2.5 mM solution of dNTPs | 8 | $\mu$l |
| | the plasmid vector described in EXAMPLE 11 (8ng/ml) | 2 | $\mu$l |
| | sterile distilled water | 73.5 | $\mu$l |
| | 20 $\mu$M solution of primer 3 | 5 | $\mu$l |
| | 100 $\mu$M solution of primer 4 (for mutagenesis) | 1 | $\mu$l |
| | Ex Taq (Takara Shuzo) | 0.5 | $\mu$l |

Specific sets of primers were used for each mutation and other components were unchanged. Primers used for the reactions are shown in Table 10. The nucleotide sequences of the primers are shown in SEQUENCE NO: 20, 23, 27 and 30-40. The PCRs were performed under the following conditions as follows. An initial denaturation step at 97℃ for 3 min was followed by 25 cycles of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 3 min. After these amplification cycles, final extension was performed at 70℃ for 5 min. The size of the PCR prodcts was confirmed by agarose gel electrophoresis using reaction solution. After the first PCR, excess primers were removed using Amicon microcon (Amicon). The final volume of the solutions that contained the PCR products were made to 50 $\mu$l with sterile distilled water. These purified PCR products were used for the second PCR (PCR 3).

| | | | |
|---|---|---|---|
| PCR 3 | 10X Ex Taq Buffer (Takara Shuzo) | 10 | $\mu$l |

| | |
|---|---|
| 2.5 mM solution of dNTPs | 8 μl |
| solution containing DNA fragment obtained from PCR 1 | 5 μl |
| solution containing DNA fragment obtained from PCR 2 | 5 μl |
| sterile distilled water | 61.5 μl |
| 20 μM solution of primer 1 | 5 μl |
| 20 μM solution of primer 3 | 5 μl |
| Ex Taq (Takara Shuzo) | 0.5 μl |

Table 10

| mutants | primer-1 | primer-2 | primer-3 | primer-4 |
|---|---|---|---|---|
| OCIF-C19S | IF 10 | C19SR | IF 3 | C19SF |
| OCIF-C20S | IF 10 | C20SR | IF 3 | C20SF |
| OCIF-C21S | IF 10 | C21SR | IF 3 | C21SF |
| OCIF-C22S | IF 10 | C22SR | IF 14 | C22SF |
| OCIF-C23S | IF 6 | C23SR | IF 14 | C23SF |

The reaction conditions were exactly the same as those for PCR 1 or PCR 2. The size of the PCR prodcts was confirmed by 1.0 % or 1.5 % agarose gel electrophoresis. The DNA fragments were precipitated with ethanol, dried under vacuum and dissolved in 40 μl of sterile distilled water. The solutions containing DNA fragments with mutation C19S, C20S, C21S, C22S and C23S were

designated as DNA solution A, DNA solution B, DNA solution C, DNA solution D and DNA solution E, respectively.

The DNA fragment which is contained in solution A (20 μl) was digested with restriction enzymes Nde I and Sph I (Takara Shuzo). A DNA fragment with an approximate size of 400 base pairs (bp) was extracted from a preparative agarose gel and dissolved in 20 μl of sterile distilled water. This DNA solution was designated DNA solution 3. Two micrograms of pSK + -OCIF [was] ^ were digested with restriction enzymes Nde I and Sph I. A DNA fragment with an approximate size of 4.2 kb was purified from a preparative agarose gel [with] ^ using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated [as] DNA solution 4. Two microliters of DNA solution 3, 3 μl of DNA solution 4 and 5 μl of ligation buffer I [of] ^ from a DNA ligation kit ver. 2 were mixed and ^ the ligation reaction was carried out. Competent [E. coli DH5 α] ^ E. coli strain DH5α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C19S.

The DNA fragment [which is] contained in solution B (20 μl) was digested with restriction enzymes Nde I and Sph I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel [with] ^ using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated DNA solution 5. Two microliters of DNA solution 5, 3 μl of DNA solution 4 and 5 μl of ligation buffer I [of] ^ from a DNA ligation kit

ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C20S.

The DNA fragment which is contained in solution C (20 $\mu$l) was digested with restriction enzymes Nde I and Sph I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel with using a QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 6. Two microliters of DNA solution 6, 3 $\mu$l of DNA solution 4 and 5$\mu$l of ligation buffer I of from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 $\alpha$ cells were transformed with 5 $\mu$l of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C21S.

The DNA fragment which is contained in solution D (20 $\mu$l) was digested with restriction enzymes Nde I and Bst PI. A DNA fragment with an approximate size of 600 bp was extracted from a preparative agarose gel with using a QIAEX gel extraction kit and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated as DNA solution 7. Two micrograms of pSK + -OCIF was were digested with restriction enzymes Nde I and Bst PI. A DNA fragment with an approximate size of 4.0 kb was extracted from a preparative agarose gel with using a QIAEX gel extraction kit and dissolved in 20$\mu$l of sterile distilled

water. This DNA solution was designated [as] DNA solution 8. Two microliters of DNA solution 7, 3 μl of DNA solution 8 and 5 μl of ligation buffer I [of] a DNA ligation kit ver. 2 were mixed and [the] ligation reaction was carried out. Competent E. coli [strain] DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA in which the 600-bp Nde I-BstPI fragment with the mutation (the C22S mutation) is substituted for the 600-bp Nde I-Bst PI fragment of pSK+ -OCIF by analyzing the DNA structure. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C22S.

The DNA fragment which is contained in solution E (20 μl) was digested with restriction enzymes Bst PI and Eco RV. A DNA fragment with an approximate size of 120 bp was extracted from a preparative agarose gel [with] [using a] QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated [as] DNA solution 9. Two micrograms of pSK + -OCIF [was] [were] digested with restriction enzymes Bst EII and Eco RV. A DNA fragment with an approximate size of 4.5 kb was extracted from a preparative agarose gel [with] [using a] QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated [as] DNA solution 10. Two microliters of DNA solution 9, 3 μl of DNA solution 10 and 5 μl of ligation buffer I [of] [from a] DNA ligation kit ver. 2 were mixed and [the] ligation was carried out. Competent E. coli [strain] DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by

DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C23S.

2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-C19S, pSK-OCIF-C20S, pSK-OCIF-C21S, pSK-OCIF-C22S and pSK-OCIF-C23S were digested with restriction enzymes Bam HI and Xho I. The 1.6 kb Bam HI-Xho I DNA fragment encoding each OCIF mutant was isolated and dissolved in 20 $\mu$l of sterile distilled water. The DNA solutions that contain 1.6 kb cDNA fragments derived from pSK-OCIF-C19S, pSK-OCIF-C20S, pSK-OCIF-C21S, pSK-OCIF-C22S and pSK-OCIF-C23S were designated C19S DNA solution, C20S DNA solution, C21S DNA solution, C22S DNA solution and C23S DNA solution, respectively. Five micrograms of [a] expression vector pCEP 4 (Invitrogen) [was] were digested with restriction enzymes Bam HI and Xho I. A DNA fragment with an approximate size of 10 kb was purified and dissolved in 40 $\mu$l of sterile distilled water. This DNA solution was designated as pCEP 4 DNA solution. One microliter of pCEP 4 DNA solution and 6 $\mu$l of either [C19SDNA] C19S DNA solution, C20S DNA solution, C21S DNA solution, C22S DNA solution or C23S DNA solution were independently mixed with 7 $\mu$l of ligation buffer I [of] DNA from a ligation kit ver. 2 and the ligation reactions were carried out. Competent E. coli strain DH5$\alpha$ cells (100 $\mu$l) were transformed with 7 $\mu$l of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmid in which a 1.6-kb cDNA fragment is inserted between the recognition sites of Bam HI and Xho I of pCEP 4 by analyzing the DNA structure. The [plasmide] plasmids which were obtained containing the cDNA encoding OCIF-C19S, OCIF-C20S, OCIF-C21S, OCIF-C22S and OCIF-C23S were designated pCEP4-OCIF-C19S, pCEP4-OCIF-C20S, pCEP4-OCIF-C21S, pCEP4-OCIF-C22S and

pCEP4-OCIF-C23S, respectively.

ii) Preparation of domain-deletion mutants of OCIF

(1) deletion mutagenesis of OCIF cDNA

A series of OCIF mutants with deletions of from Thr 2 to Ala 42, from Pro 43 to Cys 84, from Glu 85 to Lys 122, from Arg 123 to Cys 164, from Asp 177 to Gln 251 and from Ile 252 to His 326 were prepared (positions of the amino acid residues are shown in SEQ. ID NO. 4). These mutants were designated as OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2, respectively.

Mutagenesis was performed by two-step PCR as described in EXAMPLE 22-(ii). The primer sets for the reactions are shown in Table 11 and the nucleotide sequences of the primers are shown in SEQ. ID NOS. 19, 25, 40-53 and 54.

Table 11

| mutants | primer-1 | primer-2 | primer-3 | primer-4 |
|---|---|---|---|---|
| OCIF-DCR1 | XhoI F | DCR1R | IF 2 | DCR1F |
| OCIF-DCR2 | XhoI F | DCR2R | IF 2 | DCR2F |
| OCIF-DCR3 | XhoI F | DCR3R | IF 2 | DCR3F |
| OCIF-DCR4 | XhoI F | DCR4R | IF 16 | DCR4F |
| OCIF-DDD1 | IF 8 | DDD1R | IF 14 | DDD1F |
| OCIF-DDD2 | IF 8 | DDD2R | IF 14 | DDD2F |

The final PCR products were precipitated with ethanol, dried under vacuum and dissolved in 40 μl of sterile distilled water. Solutions of DNA [fragment] fragments coding for portions of OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2 were designated [as] DNA solutions F, G, H, I, J and K, respectively.

The DNA fragment [which is] contained in solution F (20 μl) was digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated DNA solution 11. Two micrograms of pSK+ -OCIF [was] were digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 4.0 kb was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated DNA solution 12. Two microliters of DNA solution 11, 3 μl of DNA solution 12 and 5 μl of ligation buffer I [of] from a DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent E. coli strain DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR1.

The DNA fragment which is contained in solution G (20 μl) was digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel [with] using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA

solution was designated as DNA solution 13. Two microliters of DNA solution 13, 3 μl of DNA solution 12 and 5 μl of ligation buffer I of a DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent E. coli strain DH5a cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR2.

The DNA fragment which is contained in solution H (20 μl) was digested with restriction enzymes Nde I and Xho I. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel using a QIAEX gel extraction kit and dissolved in 20μl of sterile distilled water. This DNA solution was designated as DNA solution 14. Two microliters of DNA solution 14, 3 μl of DNA solution 12 and 5 μl of ligation buffer I of a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli Strain DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR3.

The DNA fragment which is contained in solution I (20 μl) was digested with restriction enzymes Xho I and Sph I. A DNA fragment with an approximate size of 900 bp was extracted from a preparative agarose gel using a QIAEX gel

extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated as DNA solution 15. Two micrograms of pSK+ -OCIF was digested with restriction enzymes Xho I and Sph I. A DNA fragment with an approximate size of 3.6 kb was extracted from a preparative agarose gel with QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated as DNA solution 16. Two microliters of DNA solution 15, 3 μl of DNA solution 16 and 5 μl of ligation buffer I of DNA ligation kit ver. 2 were mixed and ligation reaction was carried out. Competent E. coli DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR4.

The DNA fragment which is contained in solution J (20 μl) was digested with restriction enzymes BstP I and Nde I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel with QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated as DNA solution 17. Two microliters of DNA solution 17, 3 μl of DNA solution 8 and 5μl of ligation buffer I of DNA ligation kit ver. 2 were mixed and ligation reaction was carried out. Competent E. coli DH5 α cells were transformed with 5μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DDD1.

The DNA fragment [which is] contained in solution K (20 μl) was digested with restriction enzymes Nde I and BstP I. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel [with] QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated [as] DNA solution 18. Two microliters of DNA solution 18, 3 μl of DNA solution 8 and 5 μl of ligation buffer I [of] DNA ligation kit ver. 2 were mixed and ligation reaction was carried out. Competent E. coli DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DDD2.

2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-DCR1, pSK-OCIF-DCR2, pSK-OCIF-DCR3, pSK-OCIF-DCR4, pSK-OCIF-DDD1 and pSK-OCIF-DDD2 were digested with restriction enzymes Bam HI and Xho I. The Bam HI-Xho I DNA fragment containing entire coding sequence for each OCIF mutant was isolated and dissolved in 20 μl of sterile distilled water. These DNA solutions that contain the Bam HI-Xho I fragment derived from pSK-OCIF-DCR1, pSK-OCIF-DCR2, pSK-OCIF-DCR3, pSK-OCIF-DCR4, pSK-OCIF-DDD1 and pSK-OCIF-DDD2 were designated DCR1 DNA solution, DCR2 DNA solution, DCR3 DNA solution, DCR4 DNA solution, DDD1 DNA solution and DDD2 DNA solution, respectively. One microliter of pCEP 4 DNA solution and 6 μl of either DCR1 DNA solution, DCR2 DNA solution, DCR3 DNA solution, DCR4 DNA solution, DDD1 DNA solution or DDD2 DNA solution were independently mixed with 7 μl of

ligation buffer I [of] DNA ligation kit ver. 2 and ligation reactions were carried out. Competent E. coli DH5α cells (100 μl) were transformed with 7 μl of each ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [a] plasmid DNA in which the DNA fragment with deletions is inserted between the recognition sites of Bam HI and Xho I of pCEP 4 by analyzing the DNA structure. The plasmids containing the cDNA encoding OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2 were designated [as] pCEP4-OCIF-DCR1, pCEP4-OCIF-DCR2, pCEP4-OCIF-DCR3, pCEP4-OCIF-DCR4, pCEP4-OCIF-DDD1 and pCEP4-OCIF-DDD2, respectively.

iii) Preparation of OCIF with C-terminal domain truncation

(1) mutagenesis of OCIF cDNA

A series of OCIF mutants with deletions [of] from Cys at amino acid residue 379 to Leu 380, from Ser 331 to Leu 380, from Asp 252 to Leu 380, from Asp 177 to Leu 380, from Arg 123 to Leu 380 and from Cys 86 to Leu 380 was prepared. Positions of the amino acid residues are shown in [SEQUENCE NO:] 4. These mutants were designated as OCIF-CL, OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3, respectively.

Mutagenesis for OCIF-CL was performed by the two-step PCR as described in EXAMPLE 22-(ii). The primer set for the reaction is shown in Table 12. The nucleotide sequences of the primers are shown in [SEQUENCE NO:] 23, 40, 55, and 56. The final PCR products were precipitated with ethanol, dried under vacuum and dissolved in 40 μl of sterile distilled water. This DNA solution was designated [as] solution L.

The DNA fragment which is contained in solution L (20 μl) was digested with restriction enzymes BstP I and EcoR V. A DNA fragment with an approximate size of 100 bp was extracted from a preparative agarose gel using a QIAEX gel extraction kit and dissolved in 20 μl of sterile distilled water. This DNA solution was designated as DNA solution 19. Two microliters of DNA solution 19, 3 μl of DNA solution 10 (described in EXAMPLE 22-(ii)) and 5 μl of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent E. coli strain DH5 α cells were transformed with 5 μl of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-CL. Mutagenesis of OCIF cDNA to prepare OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3 was performed by a one-step PCR reaction.

PCR reactions for mutagenesis to prepare OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3 were as follows:

| | |
|---|---|
| 10X Ex Taq Buffer (Takara Shuzo) | 10 μl |
| 2.5 mM solution of dNTPs | 8 μl |
| the plasmid vector containing the entire OCIF cDNA described in EXAMPLE 11 (8ng/ml) | 2 μl |
| sterile distilled water | 73.5 μl |
| 20 μM solution of primer OCIF Xho F | 5 μl |
| 100 μM solution of primer (for mutagenesis) | 1 μl |
| Ex Taq (Takara Shuzo) | 0.5 μl |

Table 12

| mutants | primer-1 | primer-2 | primer-3 | primer-4 |
|---|---|---|---|---|
| OCIF-CL | IF 6 | CL R | IF 14 | CL F |

Specific primers were used for each mutagenesis and other components were unchanged.

Primers used for the mutagenesis are shown in Table 13. Their nucleotide sequences are shown in SEQ. ID No. SEQUENCE NO:57-61. The components of each PCR were mixed in a microcentrifuge tube and PCR was performed as follows. The microcentrifuge tubes were treated for 3 minutes at 97 ℃ and then incubated sequentially, for 30 seconds at 95 ℃, 30 seconds at 50 ℃ and 3 minutes at 70 ℃. This three-step incubation procedure was repeated 25 times, and after that, the tubes were incubated for 5 minutes at 70 ℃. An aliquot of the reaction mixture was removed from each tube and analyzed by an agarose gel electrophoresis to confirm the size of each product.

The size of the PCR products was confirmed on an agarose gel. Excess primers in the PCRs were removed using an Amicon microcon (Amicon) after completion of the reaction. The DNA fragments were precipitated with ethanol, dried under vacuum and dissolved in 40 $\mu$l of sterile distilled water. The DNA fragment in each DNA solution was digested with restriction enzymes Xho I and Bam HI. After the reactions, DNA was precipitated with ethanol, dried under vacuum and dissolved in 20 $\mu$l of sterile distilled water.

The solutions containing the DNA fragment with the CC deletion, the CDD2

deletion, the CDD1 deletion, the CCR4 deletion and the CCR3 deletion were designated as CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution and CC R3 DNA solution, respectively.

Table 13

| mutants | primers for the mutagenesis |
|---|---|
| OCIF-CC | CC R |
| OCIF-CDD2 | CDD2 R |
| OCIF-CDD1 | CDD1 R |
| OCIF-CCR4 | CCR4 R |
| OCIF-CCR3 | CCR3 R |

(2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-CL was digested with restriction enzymes Bam HI and Xho I. The Bam HI-Xho I DNA fragment containing the entire coding sequence for OCIF-CL was isolated and dissolved in 20 $\mu$l of sterile distilled water. This DNA solution was designated as CL DNA solution. One microliter of pCEP 4 DNA solution and 6 $\mu$l of either of CL DNA solution, CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution or CCR3 DNA solution were independently mixed with 7 $\mu$l of ligation buffer I of DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent E. coli strain DH5$\alpha$ cells (100 $\mu$l) were transformed with 7 $\mu$l of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmids which have the

desirable mutations in the OCIF cDNA by analyzing the DNA structure. In each plasmid, the OCIF cDNA fragment having a deletion was were inserted between the recognition sites of Xho I and Bam HI of pCEP 4. The plasmids containing the cDNA encoding OCIF-CL, OCIF-CC, OCIF-CDD1, OCIF-CDD2, OCIF-CCR4 and OCIF-CCR3 were designated pCEP4-OCIF-CL, pCEP4-OCIF-CC, pCEP4-OCIF-CDD2, pCEP4-OCIF-CDD1, pCEP4-OCIF-CCR4 and pCEP4-OCIF-CCR3, respectively.

iv) Preparation of OCIF mutants with C-terminal truncation truncations

(1) Introduction of C-terminal truncation truncations to OCIF

A series of OCIF mutants with C-terminal truncation truncations was prepared. An OCIF mutant in which 10 residues of from Gln at 371 to Leu at 380 are were replaced with 2 residues of Leu-Val (Leu-Val) was designated OCIF-CBst. An OCIF mutant in which 83 residues of from Cys 298 to Leu 380 are were replaced with 3 residues of Ser-Leu-Asp (Ser-Leu-Asp) was designated OCIF-CSph. An OCIF mutant in which 214 residues of from Asn 167 to Leu 380 are were removed was designated OCIF-CBsp. OCIF muatant An OCIF mutant in which 319 residues of from Asp 62 to Leu 380 are were replaced with 2 residues of Leu-Val (Leu-Val) was designated OCIF-CPst. Positions of the amino acid residues are shown in SEQUENCE NO: SEQ. ID No. 4.

Two micrograms each of pSK + -OCIF was were digested with one of the restriction enzymes, Bst PI, Sph I, PstI (Takara Shuzo), and or Bsp EI (New England Biolabs, and followed by phenol extraction and ethanol precipitation. The precipitated DNA was dissolved in 10 $\mu$l of sterile distilled water. Ends The ends of the DNAs in 2 $\mu$l of each solution were blunted using a DNA blunting kit in a final volumes volume of 5 $\mu$l. To the reaction mixtures, 1 $\mu$g (1 $\mu$l) of an Amber

codon-containing Xba I linker (5'-CTAGTCTAGACTAG-3') and 6 μl of ligation buffer I from a of DNA ligation kit ver. 2 were added.

After the ligation reactions, 6 μl each of the reaction mixtures was used to transform E. coli strain DH5α. Ampicillin-resistant transformants were screened for clones containing plasmids. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmids thus obtained were named pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp and pSK-OCIF-CPst, respectively.

(2) Construction of vectors for expressing the OCIF mutants

pSK-OCIF-CBst, pSK-OCIF- CSph, pSK-OCIF-CBsp and pSK-OCIF-CPst were digested with restriction enzymes Bam HI and Xho I. The 1.5 kb of DNA fragment containing the entire coding sequence for each OCIF mutant was isolated and dissolved in 20 μl of sterile distilled water. These DNA solutions that contained contain the Bam HI-XhoI fragment derived from pSK-OCIF-CBst, pSK-OCIF- CSph, pSK-OCIF-CBsp and or pSK-OCIF-CPst were designated as CBst DNA solution, CSph DNA solution, CBsp DNA solution and CPst DNA solution, respectively. One microliter of pCEP 4 DNA solution (described in EXAMPLE 22-ii)) and 6 μl of either CBst DNA solution, CSph DNA solution, CBsp DNA solution or CPst DNA solution were independently mixed with 7 μl of ligation buffer I from a of DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent E. coli strain DH5α cells (100 μl) were transformed with 7 μl of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmids in which the cDNA fragment is was inserted between the recognition sites of Bam HI and Xho I of pCEP 4 by analyzing the DNA structure. The plasmids containing the cDNA encoding OCIF-CBst, OCIF-CSph, OCIF-CBsp and or OCIF-CPst

were designated as pCEP4-OCIF-CBst, pCEP4-OCIF- CSph, pCEP4-OCIF-CBsp and pCEP4-OCIF-CPst, respectively.

v) Preparetion of vectors for expressing the OCIF mutants

E. coli clones harboring the expression vectors for OCIF mutants (total of 21 clones) were grown and the vectors were purified by QIAGEN column (QIAGEN). All the expression vectors were precipitated with ethanol and dissolved in appropriate volumes of sterile distilled water and used for further manipupations shown below.

vi) Transient expression of the cDNAs for OCIF mutants and biological activities of the mutants

OCIF mutants were produced using the expression vectors prepared in EXAMPLE 22-v). The method was essentially the same as described in EXAMPLE 13. Only the modified points are described below. A 24-well plate was used for the DNA transfection. $2X10^5$ cells of 293/EBNA suspended in IMDM containing 10% fetal bovine serum were seeded into each well of the plate. One microgram of purified vector DNA and 4 $\mu$l of lipofectamine were used for each transfection. Mixture of an expression vector and lipofectamine in OPTI-MEM (GIBCO BRL) in a final volume of 0.5 ml was added to the cells in a well. After the cells were incubated at 37℃ for 24 hr in a $CO_2$ incubator, the medium was replaced with 0.5 ml of Ex-cell 301 medium (JSR). The cells were incubated at 37 ℃ for 48 more hours in the $CO_2$ incubator. The conditioned medium was collected and used for assay for in vitro biological activity. The nucleotide sequences of cDNAs for the OCIF mutants are shown in SEQUENCE NO:83-103. The deduced amino acid sequences for the OCIF mutants are shown in SEQUENCE NO:

62-82. The assay for in vitro biological activity was performed as described in EXAMPLE 13. [Antigen] concentration of each conditioned medium was determined by ELISA as described in EXAMPLE 24. Table 14 shows [specific] activity of [the mutants] relative to that of the unaltered OCIF.

Table 14

| mutants | activity |
|---|---|
| the unaltered OIF | ++ |
| OCIF-C19S | + |
| OCIF-C20S | ± |
| OCIF-C21S | ± |
| OCIF-C22S | + |
| OCIF-C23S | ++ |
| OCIF-DCR1 | ± |
| OCIF-DCR2 | ± |
| OCIF-DCR3 | ± |
| OCIF-DCR4 | ± |
| OCIF-DDD1 | + |
| OCIF-DDD2 | ± |
| OCIF-CL | ++ |
| OCIF-CC | ++ |
| OCIF-CDD2 | ++ |
| OCIF-CDD1 | + |
| OCIF-CCR4 | ± |
| OCIF-CCR3 | ± |
| OCIF-CBst | ++ |

| | |
|---|---|
| OCIF-CSph | ++ |
| OCIF-CBsp | ± |
| OCIF-CPst | ± |

++ indicates relative activity more than 50% of that of the unaltered OCIF; + indicates relative activity between 10% and 50% ± indicates relative activity less than 10%, or production level too low to determine the accurate biological activity.

vii) western blot analysis

Ten microliters of the final conditioned medium was used for western blot analysis. Ten microliters of each sample were mixed with 10 μl of SDS-PAGE sample buffer (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 20 μg/ml bromophenol blue, pH 6.8), boiled for 3 min. and subjected to a 10 % SDS polyacrylamide gel electrophoresis under non-reducing conditions. After the electrophoresis, the separated proteins were blotted to PVDF membrane (ProBlott$^R$, Perkin Elmer) using a semi-dry electroblotter (BIO-RAD). The membrane was incubated at 37°C with horseradish peroxidase labeled anti-OCIF antibodies for 2 hr. After the membrane was washed, protein bands which react with the labeled antibodies were detected using an ECL system (Amersham). Two protein bands with approximate molecular masses of 60kD and 120kD were detected for the unaltered OCIF. On the other hand, almost exclusively a 60kD protein band was detected for the OCIF-C23S, OCIF-CL and OCIF CC mutants. Protein bands with an approximate masses of 40kD-50kD and 30kD-40kD were the major ones for OCIF-CDD2 and OCIF-CDD1, respectively. These results indicate that Cys at 379 is responsible for the

dimer formation, both the monomers and the dimers maintain the biological activity and a deletion of residues from Asp at 177 to Leu at 380 does not abolish the biological activity of OCIF (positions of the amino acid resare residues are shown in SEQUENCE NO: 4). SEQ. ID No.

EXAMPLE 23

Isolation of human genomic OCIF gene

i) Screening of a human genomic library

An amplified human placenta genomic library in Lambda FIX II vector [purchased from STRATAGENE] was screened for the gene encoding human OCIF using the human OCIF cDNA as a probe. Essentially, screening was done according to the instruction manual supplied with the genomic library. The basic protocols described in Molecular Cloning: A Laboratory Manual [also were] employed to manipulate phage, E. coli, and DNA .

The library was titered, and $1x10^6$ pfu of phage was mixed with XL1-Blue MRA host E. coli cells and plated [on] 20 plates (9 cm x 13 cm) with 9 ml per plate of top agarose. The plates were incubated overnight at 37℃. Filter plaque lifts were prepared using Hybond-N nylon membranes (Amersham). The membranes were processed by denaturation in a solution containing 1.5 M NaCl and 0.5 M NaOH for 1 minute at room temperature. The membranes were then neutralized by placing [successively for one minute each] in 1 M Tris-HCl (pH7.5) and a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5). The membranes were then transferred onto a filter paper [wet] with 2xSSC. Phage DNA was fixed [on] the membranes with 1200 $\mu$ Joules of UV energy [in] STRATALINKER UV crosslinker 2400 (STRATAGENE) and the membranes were air dried. The membranes were immersed in Rapid Hybridization buffer (Amersham) and incubated for one hour at 65 ℃ before hybridization with $^{32}$P-labeled cDNA probe in the same buffer overnight at 65℃. Screening probe was prepared by labeling the

OCIF cDNA with $^{32}P$ using the Megaprime DNA labeling system (Amersham). Approximately, $5x10^5$cpm probe was used for each ml of hybridization buffer. After the hybridization, the membranes were rinsed in 2xSSC for five minutes at room temperature. The membranes were then washed four times, 20 minutes each time, in 0.5xSSC containing 0.1 % SDS at 65 ℃. After the final wash, the membranes were dried and subjected to autoradiography at -80 ℃ with SUPER HR-H X-ray film (FUJI [PFOTO→PHOTO] FILM Co., Ltd.) and an intensifying screen. Upon examination of the autoradiograms, six positive signals were detected. Agar plugs were picked from the regions corresponded to these signals for phage purification. Each agar plug was soaked overnight in 0.5 ml of SM buffer containing 1% chloroform to extract phage. Each extract containing phage was diluted 1000 fold with SM buffer and an aliquot of [1 ml or 20 ml→1μl or 20μl] was mixed with host E. coli described above. The mixture was plated [on→onto] agar plates with top agarose as described above. The plates were incubated overnight at 37 ℃, and filter lifts were prepared, prehybridized, hybridized, washed and autoradiographed as described above. This process of phage purification was applied to all six positive signals initially detected on the autoradiograms and was repeated until all phage plaques on agar plates hybridize with the cDNA probe. After purification, agar plugs of each phage isolate were soaked in SM buffer containing 1% chloroform and stored at 4 ℃. Six individual phage isolates were designated λOIF3, λOIF8, λOIF9, λOIF11, λOIF12 and λOIF17, respectively.

ii) Analysis of the genomic clones by restriction enzyme digestion and

Southern blot hybridization

DNA was prepared from each phage isolate by the plate lysate method as described in Molecular Cloning: A Laboratory Manual. DNA prepared from each phage was digested with restriction enzymes and the fragments derived from the digestion were separated on agarose gels. The fragments were then transferred to nylon membranes and subjected to Southern blot hybridization using OCIF cDNA as a probe. The results of the analysis revealed that the six phage isolates are individual clones. Among these fragments derived from [the] restriction enzyme digestion, those fragments which hybridized with the OCIF cDNA probe were subcloned into plasmid vectors and subjected to [the] nucleotide sequence analysis as described below.

iii) Subcloning restriction fragments derived from genomic clones into plasmid vectors and [determination of the] nucleotide sequence.

λ OIF8 DNA was digested with restriction enzymes EcoRI and NotI, and the DNA fragments derived [these from] were separated on a 0.7% agarose gel. The 5.8 kilobase [pairs] (kb) EcoRI/NotI fragment was extracted from the gel using a QIAEX II Gel Extraction Kit (QIAGEN) according to the procedure recommended by the manufacturer. The 5.8 kb EcoRI/NotI fragment was ligated with pBluescript II SK+ vector (STRATAGENE), which had been linearized with restriction enzymes EcoRI and NotI, using Ready-To-Go T4 DNA Ligase (Pharmacia) according to the procedure recommended by the manufacturer. Competent DH5 α E. coli cells (Amersham) were transformed with the recombinant plasmid and transformants were selected on L-plates containing 50 μg/ml of ampicillin.

A clone harboring the recombinant plasmid containing the 5.8 kb EcoRI/NotI fragment was isolated and this plasmid was termed pBSG8-5.8. pBSG8-5.8 was digested with HindIII and a 0.9 kb of DNA fragment derived from this digestion was isolated in the same manner as described above. This 0.9 kb fragment was then cloned into pBluescript II SK- at the HindIII site as described above. This recombinant plasmid containing 0.9 kb HindIII fragment was denoted pBS8H0.9.

λ OIF11 DNA was digested with EcoRI and 6 kb, 3.6 kb, 2.6 kb EcoRI fragments were isolated in the same manner as described above and cloned into a pBluescript II SK+ vector at the EcoRI site as described above. These recombinant plasmids were termed pBSG11-6, pBSG11-3.6, and pBSG11-2.6, respectively. pBSG11-6 was digested with HindIII and the digest was separated on a 0.7 % agarose gel. Three fragments, 2.2 kb, 1.1 kb, and 1.05 kb in length, were extracted from the gel and cloned independently into pBluescript II SK- vector at the HindIII site in the same manner as described above. These recombinant plasmids were termed pBS6H2.2, pBS6H1.1 and pBS6H1.05, respectively.

The nucleotide sequence of the cloned genomic DNA was determined using a ABI Dyedeoxy Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER) and a 373A DNA Sequencing system (Applied Biosystems). Plasmids pBSG8-5.8, pBS8H0.9, pBSG11-6, pBSG11-3.6, pBSG11-2.6, pBS6H2.2, pBS6H1.1 and pBS6H1.05 were prepared according to the alkaline-SDS procedure as described in Molecular Cloning: A Laboratory Manual and used as templates for the DNA sequence analysis. The nucleotide sequence of the human OCIF gene is presented in SEQ. ID NO. 104 and SEQ. ID NO. 105. The nucleotide sequence of the DNA,

between exon 1 and exon 2 was not entirely determined. There is a stretch of approximately 17 kb of nucleotides between the sequences given in SEQ. ID sequence No. 104 and SEQ. ID sequence No. 105.

EXAMPLE 24

Quantitation of OCIF by EIA

i) Preparation of anti-OCIF antibody

Male JW rabbits (Kitayama LABES Co., LTD) weighing 2.5-3.0 kg were used for in immunization for preparing antisera. Three male JW rabbits (Kitayama LABES Co., LTD) weighing 2.5-3.0 kg were used for immunization. For immunization, an emulsion was prepared by mixing an equal volume of rOCIF (200 μg/ml) and complete Freund's adjuvant (Difco, Cat. 0638-60-7). Three The rabbits were immunized subcutaneously six times at the interval of one week intervals with 1 ml of emulsion per injection. The rabbits were injected six times at the interval of seven days subcutaneously. Whole blood was obtained ten days after the final immunization and serum was separated isolated. Antibody was purified from serum as follows. Antiserum was diluted two-fold with PBS. After adding ammonium sulfate at a final concentration of 40 w/v % 40% w/v, the antiserum was allowed to stand at 4 ℃ for 1 hr. Precipitate The precipitate obtained by centrifugation at 8000 x g for 20 min. was dissolved in a small volume of PBS and was dialyzed against PBS. The resulting resultant solution was loaded onto a Protein G-Sepharose column (Pharmacia). After washing with PBS, absorbed immunoglobulin G was eluted with 0.1 M glycine-HCL buffer (pH 3.0). Elutes were The eluate was immediately neutralized with 1.5 M Tris-HCL buffer (pH 8.7) immediately and and were dialyzed against PBS. Protein

concentration was determined by absorbance at 280nm ($E^{1\%}$ 13.5).

Horseradish peroxidase labeled antibody was prepared using an ImmunoPure Maleimide Activated Horseradish Peroxidase Kit (Pierce, Cat. 31494). Briefly, one mg of IgG was incubated with 80 ug of N-succinimidyl-S-acetylthioacetate for 30 min. After deacetylation with 5 mg of hydroxylamine HCl, modified IgG was separated using a polyacrylamide desalting column. The protein pool was mixed with one mg of maleimide-activated horseradish peroxidase and incubated at room temperature for 1 hr.

ii) Quantitation of OCIF by sandwich EIA

Microtiter plates (Nunc MaxiSorp Immunoplate) were coated with rabbit anti-OCIF IgG by incubating 0.2 ug in 100 ul of 50 mM sodium bicarbonate buffer pH 9.6 at 4°C overnight. After blocking the plates by incubating for 1 hour at 37°C with 300 ul of 25% BlockAce/PBS (Snow Brand Milk Products ), 100ul of samples were incubated for 2 hours at room temperature. After washing the plates three times with PBST (PBS containing 0.05% Tween20), 100 ul of 1:10000 diluted horseradish peroxidase-labeled anti-OCIF IgG was added and incubated for 2 hours at room temperature. The amount of OCIF was determined by incubation with 100 ul of a substrate solution (TMB, ScyTek Lab., Cat. TM4999) and measurement of the absorbance at 450 nm using an ImmunoReader (Nunc NJ2000). Purified recombinant OCIF was used as a standard protein and a typical standard curve is shown in Fig. 13.

EXAMPLE 25

Anti-OCIF monoclonal antibody

i) Preparation of a hybridoma producing anti-OCIF monoclonal antibody.

OCIF was purified to homogeneity from the culture medium of human fibroblasts, IMR-90 cells by the purification method described in Example 11. Purified OCIF was dissolved in PBS at a concentration of 10 μg/100 μl. BALB/c mice were immunized by administering this solution intraperitoneally three times every two weeks. In the first and the second immunizations, the emulsion was composed of an equal volume of OCIF and Freund's complete adjuvant [was administered]. Three days after the final immunization administration, the spleen was taken out, removed and lymphocytes were isolated and fused with mouse myeloma p3x63-Ag8.653 cells according to the conventional methods conventinal method using polyethyleneglycol. Then the fused cells were cultured in HAT medium to select hybridomas hybridoma. Subsequently, to check whether the selected hybridomas produce anti-OCIF antibody, The presence of anti-OCIF antibody in each the culture medium of each hybridoma hybridomas was determined by solid phase ELISA which was prepared by coating each well of a 96-well immunoplate immunoplates (Nunc) was coated with 100 μl of purified OCIF (10 μg/ml in 0.1 M $NaHCO_3$) and by blocked blocking each well with 50% BlockAce (Snow Brand Milk Products Co. Ltd.). Briefly, The hybridoma clones secreting anti-OCIF antibody were established by limit dilution cloning 3-5 times by cloning 3 - 5 times by limit dilution and by screening using the above solid phase ELISA. Among thus obtained hybridoma clones, several hybridoma clones producing high levels of anti-OCIF several hybridoma clones with high production of anti-OCIF antibody were selected.

ii) Production of anti-OCIF monoclonal antibodies.

Each hybridoma clone secreting anti-OCIF antibody, which was obtained in

EXAMPLE 25-i) was transplanted intraperitoneally into mice given Pristane (Aldrich), at a cell density of 1 x $10^6$ cells/mouse. The accumulated ascites was collected 10 - 14 days after transplantation, thereby obtaining the transplantation and the ascites containing anti-OCIF specific monoclonal antibody of the present invention was obtained. Purified antibodies were obtained by Affigel protein A Sepharose chromatography (BioRad) according to the manufacturer's manual. Briefly, That is, the ascites fluid was diluted with an equal volume of a binding buffer (BioRad) and applied to a protein A column. The column was washed with a sufficient volume of the binding buffer and eluted with an elution buffer (BioRad). After neutralizing, the eluate obtained was dialyzed in water and subsequently lyophilized. The purity of the antibody thereby obtained was analyzed by SDS/PAGE and a homogenous band with a molecular weight of about 150,000 was detected.

iii) Selection of monoclonal antibodies having high affinity for OCIF

Each antibody obtained in EXAMPLE 25-ii) was dissolved in PBS and the protein concentration of protein in the solution was determined by the method of Lowry. Each antibody solution was diluted to with the same concentration was prepared and then serially diluted with PBS. Monoclonal antibodies, which can recognize OCIF even at highly dilute concentrations diluted solution, were selected by solid phase ELISA described in EXAMPLE 25-ii). Thus, three monoclonal antibodies A1G5, E3H8 and D2F4 were can be selected.

iv) Determination of class and subclass of antibodies

The class and subclass of the antibodies of the present invention obtained in EXAMPLE 25-iii) were analyzed using an immunoglobulin class and subclass analysis kit (Amersham). The procedure was carried out according to the protocol disclosed in the directions. The results were shown in Table 15. The antibodies of the present invention, E3H8, A1G5 and D2F4 belong to $IgG_1$, $IgG_{2a}$ and $IgG_{2b}$, respectively.

Table 15

Analysis of class and subclass of the antibodies in the present invention.

| Antibody | $IgG_1$ | $IgG_{2a}$ | $IgG_{2b}$ | $IgG_3$ | IgA | IgM | κ |
|---|---|---|---|---|---|---|---|
| A1G5 | − | + | − | − | − | − | + |
| E3H8 | + | − | − | − | − | − | + |
| D2F4 | − | − | + | − | − | − | + |

v) Determination of OCIF by ELISA

Three kinds of monoclonal antibodies, A1G5, E3H8 and D2F4, which were obtained in EXAMPLE 25-iv), were used as solid phase antibodies and enzyme-labeled antibodies, respectively. Sandwich ELISA was constructed by each combination of solid phase antibody and labeled antibody. The labeled antibody was prepared using Immuno Pure Maleimide Activated Horseradish Peroxidase Kit (Pierce, Cat. No. 31494). Each monoclonal antibody was

dissolved in 0.1 M $NaHCO_3$ at a concentration of 10 $\mu$g/ml, and 100 $\mu$l of the solution was added to each well in a 96-well immunoplate (Nunc, MaxiSorp Cat. No. 442404) followed by allowing them to stand at room temperature overnight. Subsequently, each well of the plates was blocked with 50% Blockace (Snow Brand Milk Products, Co., Ltd.) at room temperature for 50 minutes, and then was washed three times with PBS containing 0.1% Tween 20 (washing buffer).

A series of concentrations of OCIF was prepared by diluting OCIF with 1st reaction buffer (0.2 M Tris-HCl buffer, pH 7.4, containing 40% Blockace and 0.1% Tween 20). Each well of a 96-well immunoplate was filled with 100 $\mu$l of the prepared OCIF solution with each concentration, allowed to stand at 37 ℃ for 3 hours, and subsequently washed three times with the washing buffer. For dilution of POD-labeled antibody, 2nd reaction buffer (0.1 M Tris-HCl buffer, pH 7.4, containing 25% Blockace and 0.1% Tween 20) was used. The POD-labeled antibody was diluted 400-fold with 2nd reaction buffer (0.1 M Tris-HCl buffer, pH 7.4, containing 25% Blockace and 0.1% Tween 20), and 100 $\mu$l of the diluted solution was added to each well of the immunoplates. Each immunoplate was allowed to stand at 37 ℃ for 2 hours, and subsequently washed three times with the washing buffer. After washing, 100 $\mu$l of a substrate solution (0.1 M citrate-phosphate buffer, pH 4.5, containing 0.4 mg/ml of o-phenylenediamine HCl and 0.006% $H_2O_2$) was added to each well of the immunoplates and the immunoplates were incubated at 37℃ for 15 min. The enzyme reaction was terminated by adding 50 $\mu$l of 6 N $H_2SO_4$ to each well. The optical density of each well was determined at 492 nm using an immunoreader (ImmunoReader NJ 2000, Nunc).

Using three different kinds of monoclonal antibodies of antibody in the present invention, each

combination of solid phase and POD-labeled antibodies leads to accurate determination of OCIF. Each monoclonal antibody in the present invention was confirmed to recognize a different epitope of OCIF. A typical standard curve of OCIF using a combination of solid phase antibody, A1G5, and POD-labeled antibody, E3H8 is shown in Fig. 14.

vi) Determination of OCIF in human serum

Concentration of OCIF in five samples of normal human serum was determined using an EIA system described in EXAMPLE 25-v). The immunoplates were coated with A1G5 as described in EXAMPLE 25-v), and 50 $\mu$l of 1st. reaction buffer was added to each well in the immunoplates. Subsequently, 50 $\mu$l of each human serum was added to each well in the immunoplates. The immnuoplates were incubated at 37℃ for 3 hours and then washed three times with the washing buffer. After washing, each well in the immunoplates was filled with 100 $\mu$l of POD-E3H8 antibody diluted 400-fold with 2nd. reaction buffer and incubated at 37℃ for 2 hours. After washing the immunoplates three times with the washing buffer, 100 $\mu$l of the substrate solution described in EXAMPLE 25-v) was added to each well and incubated at 37℃ for 15 min. The enzyme reaction was terminated by adding 50 $\mu$l of 6 N $H_2SO_4$ to each well in the immunoplates. The optical density of each well was determined at 492 nm using an immunoreader (ImmunoReader NJ 2000, Nunc).

1st. reaction buffer containing the known amount of OCIF was treated in the same way and a standard curve of OCIF as shown in fig. 2 was obtained. Using the standard curve of OCIF, the amount of OCIF in human serum sample was

determined. The results were shown in Table 14.

Table 14

The amount of OCIF in normal human serum

| Serum Sample | OCIF Concentration (ng/ml) |
|---|---|
| 1 | 5.0 |
| 2 | 2.0 |
| 3 | 1.0 |
| 4 | 3.0 |
| 5 | 1.5 |

EXAMPLE 26

Therapeutic effect on osteoporosis

(1) Method

Male Fischer rats, 6 weeks-old, were subjected to denervation of left forelimb. These rats were assigned to four groups(10 rats/group) and treated as follows ; group A, sham operated rats without administration ; group B, denervated rats with intravenous administration of vehicle ; group C, denervated rats administered OCIF intravenously at a dose of 5 μg/kg twice a day ; group D, denervated rats administered OCIF intravenously at a dose of 50 μg/kg twice a day. After denervation, OCIF was administered daily for 14 days. After 2 weeks treatment, the animals were sacrificed and their forelimbs were dissected. Thereafter bones were tested for mechanical

strength.

(2) Results

Decrease of bone strength was observed in the animals of control groups as compared to those animals of the normal groups while bone strength was increase in the groups of animal received 50 mg of OCIF per kg body weight.

Industrial availability

The present invention provides both a novel protein which inhibits the formation of osteoclasts and an efficient procedure for producing the protein. The protein of the present invention has an activity to inhibit formation of osteoclasts. The protein will be useful for the treatment of many diseases accompanying bone loss, such as osteoporosis, and as an antigen to be used for the immunological diagnosis of such diseases.

Referring to the deposited the microorgainsm

Name and Address of the Depositary Authority

Name: National Institute of Bioscience and Human-Technology
Agency of Industrial Science and Technology
Ministry of International Trade and Industry

Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken
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Deposited date: June 21, 1995

(It was transferred from Bikkoken No. P-14998, which was deposited on June 21, 1995. Transferred date: October 25, 1995)

Acession Number: FERM BP-5267

Abstract

A protein which inhibits osteoclast differentiation and/or maturation and a method of production of the protein. The protein is produced by human embryonic lung fibroblasts and has molecular weight of about 60 kD and about 120 kD under non-reducing conditions and about 60 kD under reducing conditions on SDS-polyacrylamide gel electrophoresis, respectively.

The protein can be isolated and purified from culture medium of the said fibroblasts. Furthermore, the protein can be produced by gene engineering.

The present invention includes cDNA for producing the protein by gene engineering, antibodies having specific affinity to the protein or a method for determination of the protein concentration using the antibodies.